Pretransplant anti-MICA antibodies and Impact on

Renal allograft outcome

Dissertation submitted in part fulfilment of the requirements for the M.D. Degree Branch XXI (Transfusion Medicine and Immunohematology) examination of The Tamil Nadu Dr.M.G.R.Medical University Chennai to be held in May 2019.

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DECLARATION

This is to certify that this dissertation titled "**Pre transplant anti-MICA antibodies and impact on renal allograft outcome**" is submitted by me in partial fulfilment towards M.D. in Transfusion Medicine and Immunohematology (Branch XXI) examination of the Tamil Nadu Dr. M.G.R Medical University, to be held in May 2019.

I have independently reviewed the literature, standardized the data collection methodology and carried out the evaluation toward completion of the thesis.

Dr Blessymol Varghese
PG Registrar
Department of Transfusion Medicine and Immunohematology
Christian Medical College,
Vellore.

CERTIFICATE

This is to certify that this dissertation titled "**Pre transplant anti-MICA antibodies and impact on renal allograft outcome"** is a bonafide work done by **Dr. Blessymol Varghese** in partial fulfilment of rules and regulation from the **M.D. BRANCH XXI (Transfusion Medicine and Immunohematology)** Degree examination of the Tamil Nadu Dr. M.G.R Medical University, to be held in May 2019.

Dr. Joy John Mammen, MBBS, MD
Professor and Head,
Department of Transfusion Medicine and Immunohematology,
Christian Medical College,
Vellore.

Dr. Anna B Pulimood, MBBS, MD, PhD
Principal,
Christian Medical College,
Vellore.

CERTIFICATE

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The candidate has independently reviewed the literature, the data collection methodology and carried out the evaluation toward completion of the thesis.

Dr. Dolly Daniel, MBBS, MD

Professor, Thesis Guide & Supervisor

Department of Transfusion Medicine and Immunohematology,

Christian Medical College, Vellore

PLAGIARISM CERTIFICATE

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Document	201631051TNMGR University.docx (D42728802)
Submitted	2018-10-18 18:42 (+05:0-30)
Submitted by	Blessymol Varghese (blessbliss20@gmail.com)
Receiver	blessbliss20.mgrmu@analysis.urkund.com

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This is to certify that this dissertation work titled **"Pre transplant anti-MICA antibodies and impact on renal allograft outcome"** is of the candidate **Dr Blessymol Varghese** with registration number – **201631051** for the award of **M.D. degree in the branch of Transfusion Medicine and Immunohematology**. I personally verified the Urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains pages from introduction to conclusion and result shows two percentages (2 **%**) of plagiarism in the dissertation.

Dr. Dolly Daniel, MBBS, MD,
Professor, Thesis Guide & Supervisor
Department of Transfusion Medicine and Immunohematology,
Christian Medical College, Vellore

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ABBREVIATIONS:

MICA	Major Histocompatibility Complex class I chain-related molecule A
ACR	Acute cellular Rejection
AMR	Acute antibody mediated rejection
SAB	Single Antigen bead assay
LSA	Luminex single antigen assay
CDC	Complement Dependant Cytotoxicity
FCXM	Flowcytometric cross match
CKD	Chronic Kidney Disease
ESRD	End stage renal disease
HLA	Human Leucocyte Antigen
XmDSA	Luminex crossmatch
NBG	Normalised Background Ratio
KDIGO	Kidney Disease Improving Global Outcome
EGFR	Estimated Glomerular Filtration Rate
TREAT	Trial to Reduce Cardiovascular Events with Aranesp Therapy
ADPKD	Autosomal Dominant Polycystic Kidney Disease
CGN	Chronic glomerulonephritis
CIN	Chronic Interstitial Nephritis
RVD	Reno vascular disease.
UNOS	United Network for Organ Sharing
IDDM	Insulin-dependent diabetes mellitus

NCD	Non-Communicable Disease
NKFI	National Kidney foundation India
TBI	Total Body Irradiation
DSA	Donor-specific antibodies
NDSA	Non Donor specific antibody
MHC	Major Histocompatibility Complex
TCR	T cell receptors
NK	Natural killer cells
ELISA	Enzyme Linked Immunosorbent Assay
PRA	Panel Reactive Antibody
AECA	Anti-endothelial cell antibodies
AT1R antibodies	Angiotensin II type 1 receptor antibodies
Anti- ETAR antibodies	Endothelin-1 type A receptor antibodies
NKG2D	Natural killer (NK) group 2, member D
CMV	Cytomegalovirus
HUVEC	Human Umbilical Vein Endothelial Cells

ABSTRACT

Objectives

1. To study the prevalence of anti-MICA antibodies in the Indian patient population awaiting renal transplantation.

2. To study the impact of anti-MICA antibodies in pre-transplantation sera on renal allograft outcome in recipients who have no donor specific anti HLA antibodies.

Materials and Methods

This was a retrospective unmatched case control study including CKD patients transplanted between January 2014 through December 2017 at CMC Vellore .There were 44 controls and 44 cases included in this study. Controls were CDC crossmatch negative and Luminex DSA (XmDSA) negative renal transplant recipients who had good graft outcome. Cases were CDC cross match negative and Luminex DSA(XmDSA) negative transplant recipients who had poor graft outcome characterised by either Acute Cellular Rejection, Antibody mediated rejection, acute graft dysfunction, slow graft function or any combination of the above mentioned outcomes. Prevalence of anti-MICA antibody was assessed in patients' pre-transplantation sera using LABScreen mixed^{TM} assay from One lambda.

RESULTS

Out of a total of 88 patients 63 were Indians. The prevalence of anti-MICA antibody positivity in the overall group was 64.80 % and amongst the Indian patients was 65.1%. Pre-transplant anti-MICA antibody positivity was present in 75% of cases as opposed to 54.5% of controls (p= 0.038) indicating an association with poor graft

outcome. No correlation was observed between anti-MICA antibody positivity and history of transfusion ($p = 0.706$) or pregnancy ($p = 0.381$).

CONCLUSION

The prevalence of anti-MICA antibody positivity in pre transplant sera of Indian patients awaiting renal transplantation was higher in our study than that reported in literature. Positivity of anti-MICA antibody in pre transplantation sera is associated with poor renal allograft outcome. This finding assumes significance particularly in patients negative for anti- HLA antibodies who have poor renal allograft outcomes.

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INTRODUCTION

Renal transplantation has revolutionized the quality of care and is often the treatment of choice for patients with end-stage renal disease (1). There is much scientific data which has proven that renal transplantation is superior to long term haemodialysis with regard to long-term morbidity and mortality in patients with ESRD (2).

Transplantation is much more predictable than it was 30 years ago and innovation over the last 20 years has been rapid, delivering substantial improvement in transplant outcome. But there are key challenges which are yet to be completely addressed. Other than organ shortage , cardiovascular disease and iatrogenic added risk of infection ,one of the major barriers to a successful renal transplantation is the immunological barrier (3).The Recipient's immune system identifies the donor graft kidney as foreign and mounts an immunological attack. Though pioneers in renal transplant attempted varying methods of immunosuppression such as total body irradiation and enhanced immunosuppressants, immunological rejection of renal allografts continued to challenge.

Many causes were attributed but the most significant barriers to renal transplantation were the ABO blood group system and the HLA system. The role of anti HLA antibody in renal transplantation was described by Terasaki et al in 1968 (4). Since then various testing platforms have evolved focusing on enhancing sensitivity in the identification of these antibodies in the serum of transplant recipients. The Complement dependant cytotoxicity (CDC), Enzyme Linked Immunosorbent

Immunoassay (ELISA), Flowcytometry and Luminex are examples of such. Each platform has its unique advantages and disadvantages. Algorithms for pre transplant compatibility testing including these various platforms have significantly improved renal allograft survival. With the advent of the Luminex single antigen assay which detects the presence of donor specific anti HLA antibodies in the transplant recipient, a thorough and careful donor selection is possible which has substantially decreased the number of immunological graft rejections and adverse graft outcome.

Despite all these advances, graft rejection and poor graft function continue to challenge patients in whom no anti HLA antibodies are identified. In this setting the role of non HLA antibodies in causing graft rejection /poor graft function has been considered and the most implicated antibodies being antibodies to the MICA system, anti-endothelial cell antibodies, Angiotensin II type 1 receptor antibodies and Endothelin-1 type A receptor antibodies (5).

Of these, the Major Histocompatibility Complex class I chain-related gene A (MICA) is one of the most polymorphic and extensively studied non-HLA antigenic targets especially in renal transplantation. Though there are some conflicting reports, humoral response to MICA antigens has repeatedly been associated with inferior graft survival and an increased risk of acute and chronic rejection following kidney and liver transplantation(6). A definitive consensus on this relationship has not been arrived yet albeit there are clear indications of MICA antibodies being associated with adverse graft outcome.

Though there are studies from other parts of the world, there is very limited data on anti-MICA antibodies and its clinical impact in the Indian population. This study was done to assess the prevalence of anti-MICA antibodies in the Indian population, and the clinical impact of these antibodies when detected in pre transplant sera of patients awaiting renal transplantation.

AIM

To study the impact of anti-MICA antibodies in pre-transplantation sera on renal allograft outcome in recipients who have no donor specific anti HLA antibodies.

OBJECTIVES:

1. To study the prevalence of anti-MICA antibodies in the Indian patient population awaiting renal transplantation.

2. To study the impact of anti-MICA antibodies in pre-transplantation sera on renal allograft outcome in recipients who have no donor specific anti HLA antibodies.

MATERIALS AND METHODS

Setting:

This study was conducted in Christian Medical College, Vellore, Tamil Nadu. It is a teaching hospital providing tertiary medical care service to the residents of Vellore and surrounding districts of Tamil Nadu and some parts of Andhra Pradesh and Kerala. It also serves as a referral centre for patients from rest of India and South East Asia.

This was an unmatched retrospective case control study done in the Department of Transfusion Medicine and Immunohematology, Christian Medical College and Hospital, Vellore, India. The study was approved by the Research and Ethics committee of the Institutional Review Board, Christian Medical College, Vellore (IRB Min No: 10464 dated on 05.01.2017).

The study required a total of 88 pre transplantation sera samples (44 cases and 44 controls) from patients who underwent renal transplantation at Christian Medical College Vellore. Both cases and controls were recruited retrospectively till the sample size was reached. All cases and controls had undergone renal transplantation at CMC Vellore from January 2014 through December 2017. Patients' pre transplantation sera were stored in HLA laboratory and were available for the study purpose with the permission of the Department of Nephrology.

SAMPLE SIZE:

Sample size calculation was done based on the study by Sumitran et al.(7).

Probability of exposure in control group	0.1
Anticipated odds ratio	5
Probability of exposure in case group	0.32
Power (1- beta) %	82
Alpha error (%)	5
1 or 2 sided	2
Required sample size in each of the case && control groups	41

Table 1: Sample calculation

Cases and controls were defined as below:

1) CONTROLS:

Controls were defined as CDC cross match negative and Luminex DSA(XmDSA) negative renal transplant recipients who had good graft outcome**.**

2) CASES:

Cases were defined as CDC cross match negative and Luminex DSA(XmDSA) negative renal transplant recipients who had poor graft outcome characterised by either biopsy proven Acute cellular rejection (ACR), Antibody mediated rejection

(AMR), Acute graft dysfunction , Slow graft recovery or any combination of the above mentioned outcomes

Graft outcomes were defined as follows.

AMR

The following biopsy criteria had to be fulfilled for classifying a case as AMR (8).

1. Histologic evidence of acute tissue injury, including one or more of the following:

- Microvascular inflammation (g >0 in the absence of recurrent or de novo glomerulonephritis, and/or ptc >0)
- Intimal or transmural arteritis (v >0)1
- Acute thrombotic microangiopathy in the absence of any other cause
- Acute tubular injury in the absence of any other apparent cause

2. Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:

- Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections or C4d >0 by IHC on paraffin sections)
- At least moderate microvascular inflammation ([g + ptc] ≥2), although in the presence of acute TCMR, borderline infiltrate, or infection; ptc ≥2 alone is not sufficient, and g must be ≥1

ACR

Various grades of ACR based on histologic characteristics are included under ACR groups.

IA. Significant interstitial inflammation (>25% of non-sclerotic cortical parenchyma, i2 or i3) and foci of moderate tubulitis (t2)

IB. Significant interstitial inflammation (>25% of nonsclerotic cortical parenchyma, i2 or i3) and foci of severe tubulitis (t3)

IIA. Mild to moderate intimal arteritis (v1) with or without interstitial inflammation and tubulitis

IIB. Severe intimal arteritis comprising >25% of the luminal area (v2) with or without interstitial inflammation and tubulitis

III. Transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)(9).

Acute Graft Dysfunction (9)

Presence of one of the following in renal allograft was defined as acute allograft dysfunction

- Rise in serum creatinine of ≥25 % from baseline within a one-to-three-month time period post transplantation.
- Failure of serum creatinine to decrease following transplantation
- Proteinuria >1 g/day.

Slow graft function (9)

Inability to attain target creatinine by the end of first week post transplantation is defined as slow graft function.

Inclusion Criteria:

1) CASES

Renal transplant recipients with

- A negative pre transplant CDC crossmatch and a negative pre transplant Luminex DSA (XmDSA). (Mean Fluorescent Intensity (MFI) <1000 was considered negative)
- Poor graft outcome characterised by biopsy proven AMR, ACR, clinically slow graft recovery, acute graft dysfunction or a combination of the above.

2) CONTROLS

Renal transplant recipients with

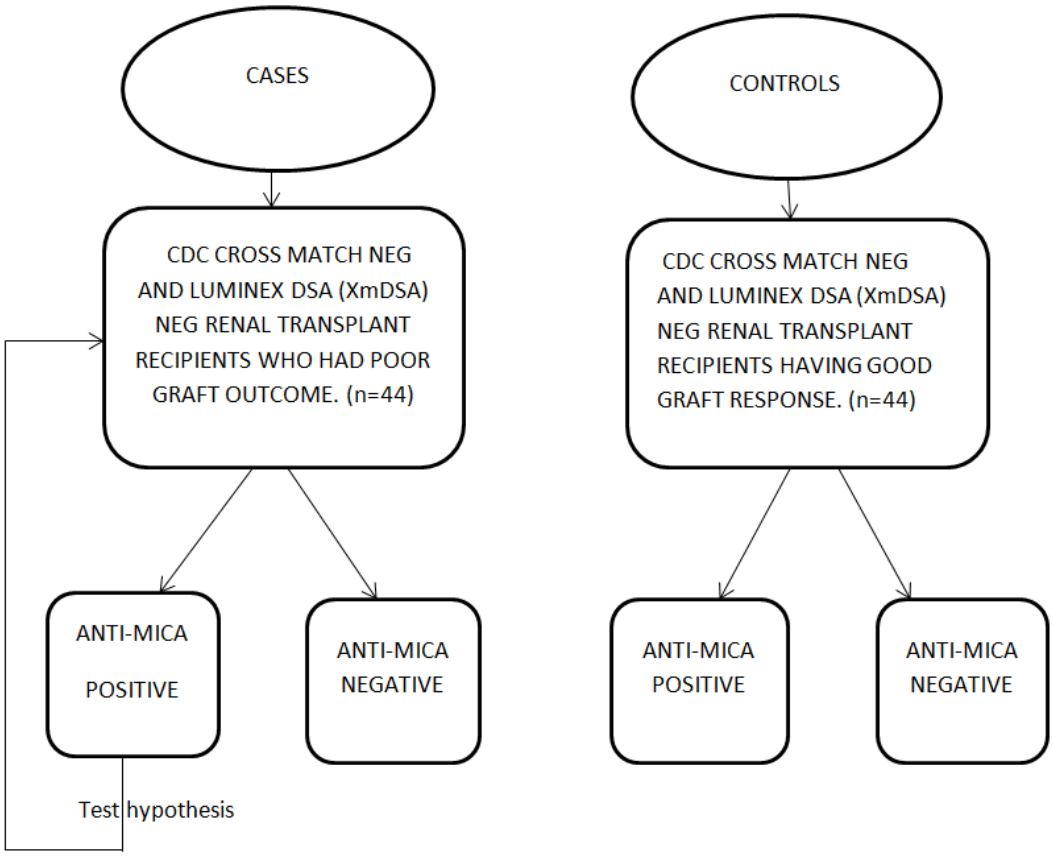
A negative pre transplant CDC crossmatch and a negative pretransplant Luminex DSA (XmDSA) having stable graft outcome.

Exclusion Criteria:

Renal transplant recipients with

- A positive Pre transplantation CDC crossmatch
- A positive HLA Class I and Class II Luminex DSA (XmDSA) in pretransplant sera. (MFI of >1000).

STUDY ALGORITHM



STUDY IMPLEMENTATION:

All patients who fit inclusion criteria from Jan 2014 to Dec 2017 were included in this study. Clinical details of the patients were collected from the patient transplant records at Department of Nephrology, CMC Vellore and hospital clinical workstation. A minimum of 41 cases and 41 controls were required for the study as per IRB approval, but we could enrol 44 cases and 44 controls fulfilling all criteria. After patients were shortlisted, their pre transplantation sera stored at -80 degree C, were identified from the HLA laboratory archived sample register and retrieved. Samples were thawed and ultra-centrifuged (8,000 – 10,000 g for 10 minutes) to remove aggregates and back

ground noise. Anti-MICA antibody testing was performed using the LABSCREEN™ mixed assay. The **LABSCREEN™ mixed Catalogue #LSM12 lot 021 was used for the study.**

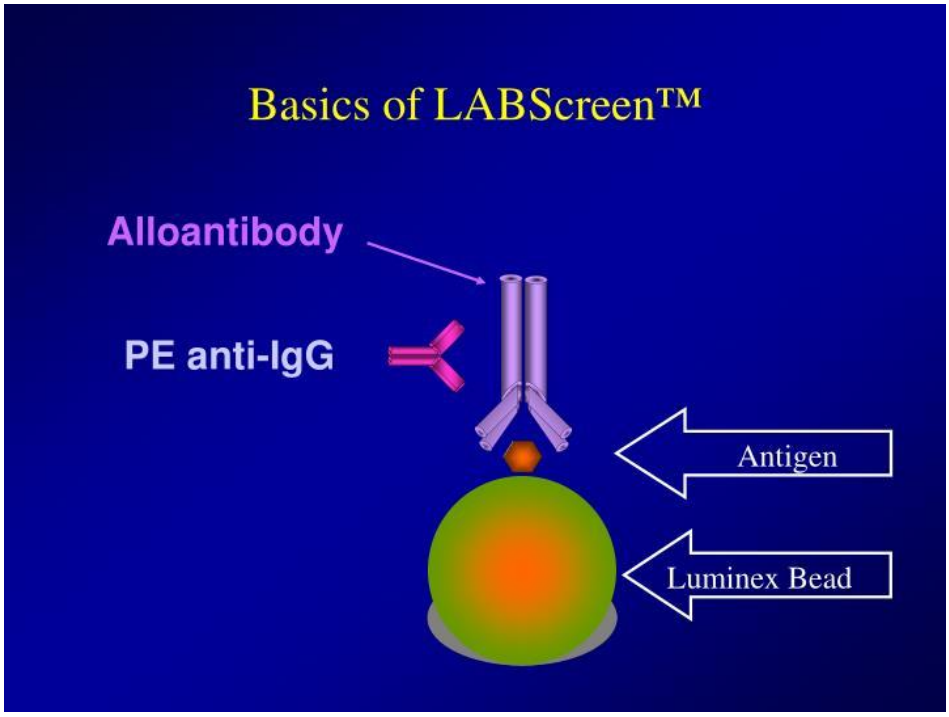
LABSCREEN™ mixed assay

LABSCREEN™ mixed assay manufactured by One lambda (A Thermo Fischer scientific brand) uses micro beads which are differentially coated with purified MICA and HLA antigens (111) and pre-optimized reagents. This assay detects antibody bound MICA antigen coated beads differentially to distinguish between anti-MICA and anti HLA antibodies in human sera.

PRINCIPLE:

The test serum is incubated with LABScreen beads which are coated with purified MICA and HLA antigens. This is followed by addition of a secondary antibody (R-Phycoerythrin (PE)-conjugated goat anti-human IgG). If anti-MICA or anti HLA antibodies are present , they will bind to the antigens pre coated on the beads and subsequently will bind to the labelled secondary antibody. The samples are then analysed in LABScan3D™ flow analyser which simultaneously detects the fluorescent emission of PE and dye signature from each bead, allowing almost real-time data acquisition.

FIGURE 1



Materials:

1. PE–Conjugated Goat Anti-Human IgG (OLI Cat. # LS-AB2)

2. PBS, filtered [USA Scientific Cat. # 9242 (500 ml 10X) or equivalent]

3. 1.5 ml micro centrifuge tube (USA Scientific Cat. # 1415-2500 or equivalent)

4. Rainin GPS Pipette tips

5. Negative Control Serum, containing no HLA antibody when tested by LABScreen method (OLI Cat. # LS-NC or equivalent)

6. 96-well micro plate, 250 µl, non-treated surface (Whatman Cat. # 7701-3250 or equivalent)

7. Tray seal

8. LAB Screen® Mixed Bead Mix (Cat. # LSM12BD)

9. LAB Screen® Wash Buffer- 10X

10. Testing sample which is ultra-centrifuged

Equipment:

1. LABScan3DTM flow analyser (Luminex® FLEXMAP 3D®) with XY platform and sheath fluid delivery system (OLI Cat. #LABSCNXS4) which is calibrated

2. Centrifuge

3. Rotor for 1.5 ml micro centrifuge tube (9,300 g),

4. Vortex mixer

5. Plate shaker

Software:

1. HLA Fusion TM (OLI Cat. # FUSPGR) for analysis

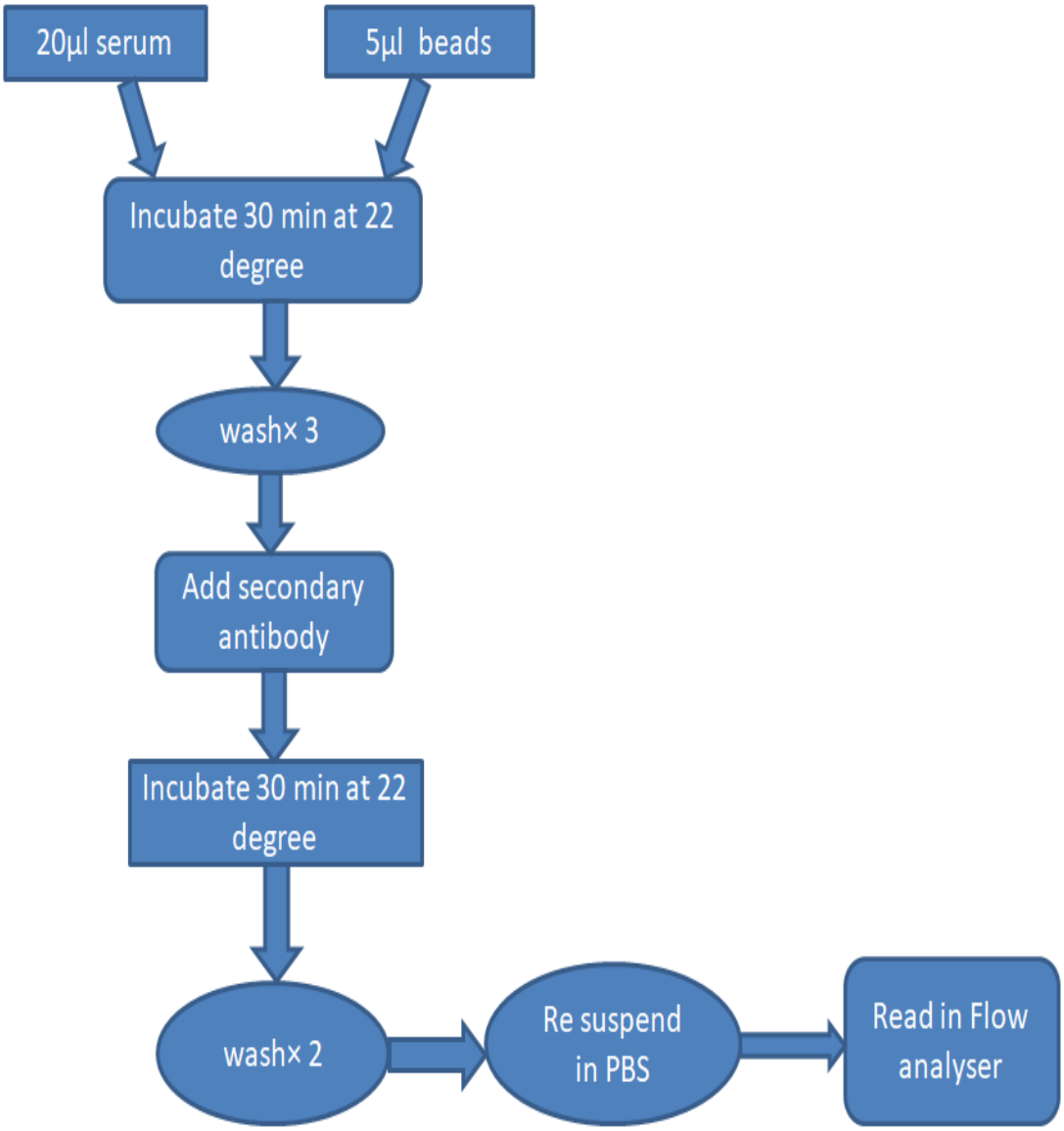
2. XPONENT for sample run

TEST PROCEDURE:

LABScan3D^{TM} flow analyser was turned on at least 30 minutes before starting the assay and the machine maintenance was done if required. A filename and sample code sheet for each test tray was created. Special care was taken in the aliquoting process, failure of which may result in reagent loss. For each test batch, a negative control

serum which is catalogue and lot specific (Catalogue #LSM12lot021) is used to establish background values.

- LAB Screen beads are mixed well by gently vortexing or pipetting up and down several times prior to use.
- 5 µl of LAB Screen beads are incubated with 20 µl of test serum in a 1.5 ml micro-centrifuge tube for 30 minutes, in the dark at 20 – 25ºC with gentle shaking.
- 10X wash buffer (OLI Cat. # LSPWABUF) which came in the kit is diluted in distilled water to make a 1X solution.
- 1 ml of 1X wash buffer is added to each bead/serum solution tube and vortex. Centrifuged at 9,300 g for 2 minutes. Supernatant is discarded. This wash step is performed twice carefully without any bead loss.
- Now Diluted PE-conjugated anti-human IgG is prepared.1 µl per test of 100 X PE-conjugated anti-human IgG (OLI Cat. # LS-AB2) is diluted with 99 µl of 1X wash buffer to make a 1X solution.
- 100 µl of 1X PE-conjugated anti-human IgG is added to each tube. Vortex and then incubated in the dark for 30 minutes at 20 - 25o C with gentle shaking.
- Washing with 1 ml of 1X wash buffer step was performed twice to wash off the unbound conjugate.
- 80 µl 1X PBS is added to each tube and proceeded to data acquisition and analysis. This can be stored at 2 - 8o C in the dark for up to 24 hours before analysis.



Algorithm of steps in LABScreen Mixed Assay

DATA ACQUISITION

- LABScan 3DTM flow analyser was already set for sample acquisition and was calibrated according to the Luminex User's Manual(10).
- Template is chosen according to product kit catalogue ID and lot number.

(Acquisition templates are available from One Lambda Inc (OLI) by CD or via download website) Luminex software versions LAB Scan 3D (xPONENT 4.2) is used. LAB Scan 100(xPONENT 4.2) can also be used.

- A file name is created for the batch of samples to be run.
- Template specifications are:

a. Set sample volume to 50 µl.

b. Set sample time-out to 80 seconds.

c. Set doublet discriminator gate to 8,000 (low limit) and 16,000 (high limit).

d. Set number and ID of beads selected according to the product-specific worksheet provided with the product.

e. Set minimum events collected to 100 per bead.

- Each sample ID is entered (if the same sample is tested more than once, assign a different ID).
- The Millipore plate is loaded onto the XY platform and the reservoir is filled with sheath fluid.
- The START button is clicked to initiate the session. After the samples have finished running, the output data is saved in a .csv file.
- The machine is washed twice with sheath fluid at the end of the session.

Data Analysis

- Data was analysed in HLA Fusion software. The reactivity of a test sample is calculated from the "raw" fluorescence values recorded by the LAB Scan device (.csv file) for each MICA as well as HLA coated bead.
- Anti-MICA antibody reactivity in the serum is calculated by correcting for non-specific binding to the negative control bead and background values (obtained by testing with a negative control serum (OLI Cat. # LS-NC) to determine the normalized background ratio (NBG ratio). The fluorescent signal (value) can be either the trimmed mean or median value.

Calculations

NBG ratio = (S#N - SNC bead)/ (BG#N - BGNC bead)

NBG	Ratio Normalized Background ratio used to assign strength of each anti-HLA reaction
S#N	Sample-specific fluorescent value for bead #N
SNC bead	Sample-specific fluorescent value for Negative Control bead
BG#N	Background NC Serum fluorescent value for bead #N
BGNC	Background NC Serum fluorescent value for Negative Control bead

bead	
NC Serum	Negative Control Serum (OLI Cat. # LS-NC) validated for a given lot of LABScreen beads

If (BG#N-BGNC bead) <50 then use 50 as a default threshold value.

After every run summary of the batch run can be generated .This will give overall result of the test samples.

The Interpretation of results for the LABScreen®Mixed assay is as shown below:

NBG Ratio	**Interpretation**
>1.5	Positive
1.2-1.5	Undetermined
<1.2	Negative

Raw data for each processed sample can be visualised which shows the reactivity pattern of the tested sample against each antigen coated bead and their interpretation (Positive, Negative or undetermined). Each test's result in the form of NBG ratio can be seen as bar histogram which will be red in colour for positive results, green in colour for negative results and grey in colour for undetermined results.

- Each sample bead count should be over 50. A lower bead count can be due to bead loss during the washing steps or improper calibration or clogging of the LABScan™100 or LABScan3D™ flow analyser, or by photo-bleached beads that dropped out from the mapped region.

- Signal values are the fluorescence intensity of each bead set against the test serum. A negative control serum is tested with the same batch of samples to establish the background value(s) for that test run.
- Negative Control Serum used with each run is One lambda Inc catalogue and lot specific (OLI Cat. # LS-NC or equivalent).
- Negative Control Beads are not coated with HLA or MICA antigens. The fluorescence value of the NC bead can vary among different sera due to non-specific binding of the sera or to insufficient washing. The NC value is usually of a MFI< 500 except for those serum samples which are having a high background. NC should always be lower than MFI of 1500and less than or equal to half of the PC value.
- Positive Control Beads are already coated with purified human IgG, which binds to the secondary antibody to produce a positive signal. The PC beads value should be over 500 and at least twice the NC value.

Validation of the Assay

For a given serum, run is valid if the value for PC/NC is greater than 2. A lower value can be due to an extremely high NC bead background value for the test serum, a high HLA bead signal for the NS control, or a low signal from the secondary antibody or flow analyser. In such case, the data have to be confirmed. **We processed our samples in 4 batches. Each batch run included a NC serum. All runs were valid with PC/NC ratio of >2.**

PRECAUTIONS TO BE TAKEN

Sera or plasma samples containing aggregates or contaminants can clog the LAB Scan flow analyser and generate inaccurate data. Hence the test specimen should be centrifuged or filtered prior to testing.

- The presence of IgG-IgM immune complex may cause inhibition in some patient samples. Samples should be treated to reduce this presence according to the protocols determined by the laboratory, however, samples should not be heat treated as they may cause non-specific background(11)
- Ambient temperature may affect LABScan 100™and LABScan3D™ performance. If the ambient temperature changes, the machine may need to be re-calibrated.
- The LABScan 100™and LABScan3D™ flow analyser must be properly calibrated and maintained. If insufficiently flushed, aggregates of the sample may cause the machine to clog and generate invalid data.

Statistics:

Data entry was performed in Microsoft excel sheet. Data was analysed using Microsoft excel and IBM SPSS Statistics 21 software. Frequencies and percentages were performed for presenting all categorical variables. Pie charts and bar charts were used for graphical representation. Pearson's Chi square test was used for finding statistical significance and p value <0.05 is considered as statistically significant. The risk factor analysis was performed using logistic regression analysis. Odd's ratios with 95% CI were presented.

REVIEW OF LITERATURE

Kidneys play a very important role in the normal functioning of our body.

- They help to maintain a constant extracellular environment that is required for adequate functioning of the cells. This is achieved by excretion of the waste products of metabolism such as urea, creatinine, and uric acid. They do this by specifically adjusting the urinary excretion of water and electrolytes to match net intake and endogenous production.

- They play a very important role in the normal electrolyte maintenance of our body. By regulating tubular reabsorption and secretion the kidney is able to regulate individually the excretion of water and solutes such as sodium, potassium, and hydrogen.

- They secrete hormones that participate in the regulation of systemic and renal hemodynamic homeostasis (renin, prostaglandins, and bradykinin), erythropoiesis (erythropoietin), and calcium, phosphorus, and bone metabolism (1, 25-dihydroxyvitamin D3 or calcitriol).

When kidneys are affected some or all of these functions may be diminished or entirely absent thereby resulting in the retention of uremic toxins, marked abnormalities in fluid and electrolyte balance, anaemia and bone disease.

Chronic Kidney Disease

Chronic Kidney disease (CKD) is a condition where kidneys lose their functioning capacity gradually over months to years. According to KDIGO 2017 Clinical Practice

Guideline Update for the Diagnosis, Evaluation, Prevention, and Treatment of Chronic Kidney Disease–Mineral and Bone Disorder **"CKD is defined as abnormalities of kidney structure or function present for more than 3 months with implications for health"**(12).

Chronic kidney disease is classified into five stages based on the level of urinary protein excretion and renal function as measured by the estimated glomerular filtration rate (eGFR). eGFR can be derived from age, race, sex, and serum creatinine concentration. End-stage renal disease (ESRD) is classified as CKD stage 5(13).

DEFINITION OF ESRD

According to Robbins and Cotrans pathological basis of disease, ESRD is defined the stage where all four morphological components of kidney which include the glomeruli, tubules, interstitium, and blood vessels are damaged due to chronic kidney disease (14).

As per another definition based on The Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) "ESRD is decrement in the subject's kidney function to a level at which either dialysis or kidney transplantation is required to sustain life meeting one of the following:

(i) Underwent >30 days of dialysis therapy

(ii) Received a kidney transplant

(iii) A physician recommended renal replacement therapy (RRT) (dialysis and/or transplant) and the subject refused therapy

(iv) Began dialysis and expired <30 days later"(15).

End-stage renal disease (ESRD) corresponds to an eGFR of <15mL/min/1.73m^2 (16), initiation of maintenance dialysis or receipt of pre-emptive renal transplantation (17).

When chronic kidney disease progress to ESRD patients develop signs and symptoms of Uraemia. At that point it is necessary that patient should receive renal replacement therapy (RRT) in the form of haemodialysis or peritoneal dialysis or renal transplantation for reversal of uraemia and survival.

GLOBAL BURDEN OF END STAGE RENAL DISEASE

Worldwide, an estimated 200 million people have chronic kidney disease (CKD) (17). Beginning from CKD stage 3, overall mortality and cardiovascular-specific mortality increases to three to 13 times higher than that of the general population and, by the onset of ESRD, the annual death rate is 17% to 20% (18).

The burden of CKD continues to increase on a global level (19).With improved sanitation and immunization programmes initiated low- to middle-income countries are undergoing epidemiologic transition to a relative increase in the burden of non-communicable chronic diseases such as diabetes mellitus, obesity, hypertension, cardiovascular disease, CKD etc. and a decrease in infectious disease prevalence (20,21). Globally a combined population >1 billion in more than 100 countries have no provisions for chronic maintenance dialysis or kidney transplantation and therefore more than 1 million people die every year from ESRD (21,22). Chronic kidney disease imposes not only incalculable human suffering but also dangerous economic burden on the patient and society. Only a fraction of the percentage of patients having ESRD, in developing countries, is able to afford or are getting the access to renal

replacement therapy. Increasing prevalence of hypertension and diabetes mellitus in younger population lead to early progression of chronic Kidney disease to ESRD.

Chronic Kidney Disease burden in India

India is the second most populous country in the world. As the life expectancy and prevalence of lifestyle diseases like hypertension and Diabetes are on the rise prevalence of CKD in India is increasing just like the western world. It is estimated that over 40% of all deaths in India are due to non-communicable disease (NCD). The exact burden of CKD or ESRD in India is not known as there is very limited data. Table 1 compares various studies published on the prevalence of CKD in various parts of India (23).

Authors	*n*	Mean age (in years)	DM (%)	HTN (%)	Criteria of CKD	Stage and prevalence	Limitations
Agarwal *et al.*	4712 urban	42±13	10.7	22.13	Creatinine ≥1.8 mg/dl on two occasions 8-12 weeks apart	Stage 3 and above=0.785%	Creatinine cut off 1.8 mg/dl is high Proteinuria subset not included as CKD GFR not calculated
Varma *et al.*	3398 mixed	35.65±8.72	1.53	15	Almuminuria >30 mg/L or albumin/creatinine ratio >30 and/or GFR <60 ml/min by MDRD and CKD-EPI	Stage 1-3=13%	Albuminuria done only once
Singh *et al.*	5588 urban	45.2±15.2	18.8	43.1	Dip stick proteinuria GFR <60 ml/min	Stage 1-5=17.2%, 6% stage 3 and above	Voluntary approach Large number having DM and HTN (high-risk group), population not true representative
Singh *et al.*	5252 semi-urban	-	7.3	31.2	Dip stick proteinuria and GFR <60 ml/min	Stage 3 and above=4.2%	Proteinuria not repeated

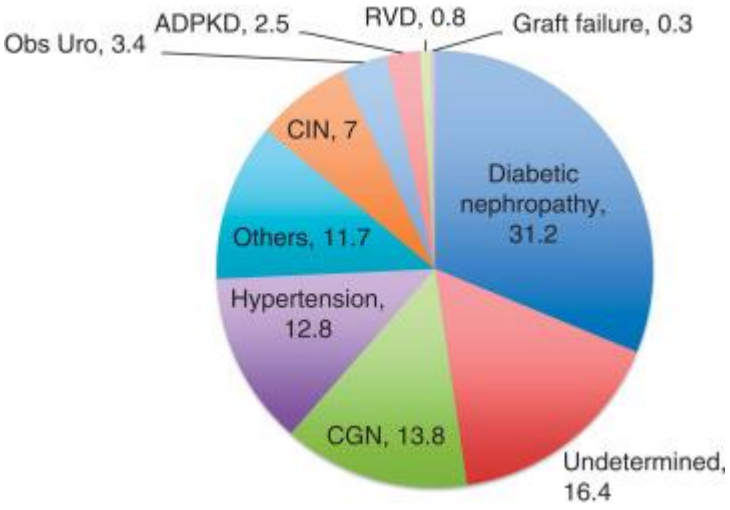
DM: Diabetes mellitus, HTN: Hypertension, CKD: Chronic kidney disease, GFR: Glomerular filtration rate, MDRD: Modification of diet in renal disease, CKD-EPI: Chronic kidney disease epidemiology

Table 2 Prevalence of CKD in India (23)

A population-based study calculated the end-stage renal disease (ESRD) incidence in India as 152 per million populations. In India Diabetic Kidney disease is the commonest cause of ESRD. It is observed that CKD of undetermined aetiology forms a large proportion as well (24).

Figure 2

Causes of end-stage kidney disease indicated as percentage in India(25)



ADPKD, Autosomal dominant polycystic kidney disease; CGN, chronic glomerulonephritis; CIN, chronic interstitial nephritis; RVD, Reno vascular disease.

There is a huge gap in distribution of Nephrology services available between the private and public health sectors as greater part of nephrology services are concentrated in expensive private sector hospitals. For a large section of our population economic issues limit the availability of renal replacement therapy like haemodialysis and peritoneal dialysis. Lack of optimization of dialysis prescription is another major concern. While renal transplant is the most suitable option for a majority of patients, most of the time it is dependent on living donors in our country.

The government of India has included kidney disease as one of the priority areas. Setting up facilities to provide subsidized dialysis for the population is under

consideration. We have a national transplant registry and organ transplant program for procurement of organs(24).

According to the National Kidney foundation India (NKFI) (http://www.nkfi.in)

- Kidney diseases rank 3rd amongst life threatening diseases after cancer and cardiac ailments.
- 100/ million population of India succumb to kidney diseases
- 90000 kidney transplants are required per year in India.
- Of patients requiring dialysis only 22.5% are receiving treatment.
- Of patients requiring transplants only 2.5% receive transplants which are live related transplants
- Due to medical, social and financial reasons only 25% of willing donors are suitable for donation.

CAUSES OF END-STAGE RENAL DISEASE WHICH CAN BE MANAGED BY RENAL TRANSPLANTATION

Based on data from the renal-transplantation registry maintained by the United Network for Organ Sharing (UNOS), which contains records of 21,161 first cadaveric transplantations reported causes of end-stage renal disease as follows

DIAGNOSIS	PERCENTAGE
IDDM	31%
Chronic glomerulonephritis	28%
Polycystic kidney disease	12%
Nephrosclerosis	09%
systemic lupus erythematous	03%
interstitial nephritis	03%
IgA nephropathy	02%
Alport's syndrome	01%

Table 3. Reported causes of ESRD requiring transplantation

Renal transplantation: Historical aspects

- The first successful human renal transplantation was done by Dr.Joseph Murray at Boston in 1954, bypassing the barrier of rejection using the patient's identical twin as the donor (26,27). This success gave sheer motivation and enthusiasm among other surgeons to pursue renal transplantation with greater confidence.
- In 1958, Murray's team conditioned two human kidney recipients with lethal total body irradiation (TBI) and donor bone marrow. Ten other recipients were treated with sublethal TBI without bone marrow. Of these 12 recipients only one survived who was not given bone marrow and he had maintained adequate function of his fraternal twin brother's kidney for 20

years. Disappointingly, rest all 11 irradiated patients died within a month. Scientifically, this was a much more important accomplishment than the identical twin case transplantation because it was the first time the genetic barrier to human renal transplantation had been breached (28).

- Later in the 1960s Gertrude Elion and George Hitchings invented 6-MP and Azathioprine as immunosuppressants which was another milestone in attaining therapeutic immunosuppression. Soon these drugs were increasingly used for renal transplantation(29).

Transplantation Immunology: Historical overview

Cellular theory of rejection

From the early era of organ transplantation, medical science has seen many graft rejections and the main question has been whether grafts were rejected by antibodies or by cells. For almost 40 years the "cellular theory" was the only theory which was accepted as correct. Sir Peter Medawar was the personality behind the Cellular theory of rejection. He is considered as the central figure in the field of transplantation. Sir Medawar was a young Oxford zoologist who was assigned to join plastic surgeon Thomas Gibson in exploring the use of skin homograft for treatment of burned aviators in World War II. While working in the Burn Unit of Glasgow's Royal Infirmary, they observed that skin homografts always failed. They attributed this rejection to an immunological phenomenon. Being a zoologist, while working on cattle, he had observed that fraternal twin cattle accept skin homografts without mounting immunological rejection. Sir Medawar reasoned out that shared intra uterine

transfusion among fraternal twins caused sharing of stem cells, red cells and leucocytes resulting in donor chimerism. He worked on induction of donor chimerism in mice and homograft tolerance and was awarded the Nobel Prize in 1966 (30).

Humoral theory of rejection

In 1938 P.A Gorer demonstrated that haemagglutinating antibodies appeared concomitantly with the rejection of tumour homograft in mice. That was the first time

Where the transplantation community started contemplating on the role of antibodies in transplant rejection (31). Based on emergent studies on humoral immunity in transplant rejection, several groups investigated their role in transplantation discovering anti-HLA antibodies. In 2003, Dr.Paul Terasaki showed adequate proof supporting the "humoral theory" of transplantation. In his article he described the association of anti-HLA antibodies detection with renal allograft rejection, both acute and chronic. (32).

RENAL ALLOGRAFT DYSFUNCTION

Immunosuppressant drugs and their development has been a huge milestone in the world of transplantation, and has greatly impacted on allograft survival. Since the introduction of Calcineurin inhibitors and anti-proliferative agents there has been a significant reduction in the incidence of acute renal allograft rejection in the past three decades. However just like the two sides of a coin the immunosuppressants carry their own price in terms of drug toxicity, dyslipidaemia, new onset diabetes, infection and increased risk of malignancy. Hence optimization of immunosuppression is a challenge indeed.

However acute graft rejection continues to be seen and is a cause of renal allograft dysfunction. Despite being under the umbrella of maximal anti-rejection therapy, some allografts have poor outcomes, and poor graft function. While these patients mostly recover from the acute rejection episode, its negative impact on the long term survival of the graft is significant. Chronic allograft nephropathy is responsible for the most death censored graft loss after the first year transplant. Acute rejection is a major predictor of chronic allograft nephropathy (33).

Types of Renal allograft rejections

On the basis of the underlying mechanisms, morphology and the timing rejection reactions are classified as hyper acute, acute, and chronic(14).

Hyper acute Rejection:

It can occur within minutes or hours after transplantation and the rejected allograft kidney rapidly becomes cyanotic, flaccid, and mottled. It is due to preformed donor specific antibodies and the graft has to be removed immediately.

Acute renal allograft rejection

It is as an acute deterioration in graft function associated with specific pathologic changes in the graft biopsy. It can occur any time within days of transplantation to 3 months afterward. In this complex process the preformed antibodies or infiltrating cells of the host immune system implicate allograft injury.

Based on histology there are two principal forms of acute rejection:

- **Acute Cellular rejection** (T Cell Mediated Rejection)

  It is characterized by lymphocytic infiltration of the tubules, interstitium and in some cases, the arterial intima.

- **Acute antibody-mediated rejection** (ABMR):

  It is characterized by morphologic evidence of acute tissue injury, circulating donor-specific alloantibodies (DSA), and immunologic evidence of an antibody-mediated process (C4d deposition in the allograft).

Chronic Rejection:

Chronic rejection presents with progressive renal failure manifested by a rise in serum creatinine over a period of 4 to 6 months. It is dominated by vascular changes (14).

The Human Leukocyte Antigen

The first HLA antigen was detected by Jean Dausset in 1958. He studied sera of multiply transfused individuals and identified seven sera that behaved similarly, in that they agglutinated leucocytes from 11 of 19 individuals tested. He gave the name 'MAC' to this antigen to honour three volunteers of his experiments and whose names began with the initials M, A and C, respectively (34). Antigen MAC was later identified as HLA –A2. He was awarded the Nobel Prize in 1988 for this discovery. From that point through contributions of several great minds and via many

international histocompatibility workshops HLA system, its complexities and sheer importance in immunogenetics have grown to today's dimension.

HLA as Human Transplantation antigens

In the early 1960s, both Dausset and co-workers and van Rood and co-workers identified that the HLA antigens are strong histocompatibility antigens. They observed that skin grafts between HLA identical siblings who inherited identical HLA haplotypes from both parents had a significantly longer graft survival than those siblings who had difference in one or both HLA haplotypes (35).

The first data on HLA matching and renal allograft survival was by Paul Terasaki in 1968(36) .The impact of HLA matching on clinical kidney transplantation was studies by Brent (37) and Kissmeyer-Nielsenand Thorsby (35) in the same period. The results of skin grafting and kidney grafting on HLA matched individuals paved the way for identification of HLA as the major histocompatibility antigenic system of humans. At the start of 1970's many centres started implementing HLA typing between donor and recipient for renal transplantation understanding the importance of HLA matching and its impact of renal allograft survival. In 1978 three independent groups demonstrated the impact of DR matching in cadaveric renal transplantation (38–40). Soon many centres started considering HLA A, B and DR typing as the major factor determining histocompatibility in transplantation.

STRUCTURE OF HLA

The HLA Complex is located on chromosome 6. (Figure 2) .It contains over 200 genes, out of which more than 40 genes encode leucocyte antigens. The HLA genes

which are important in the human immune response are class I and class II. These 2 classes of HLA genes are structurally and functionally different.

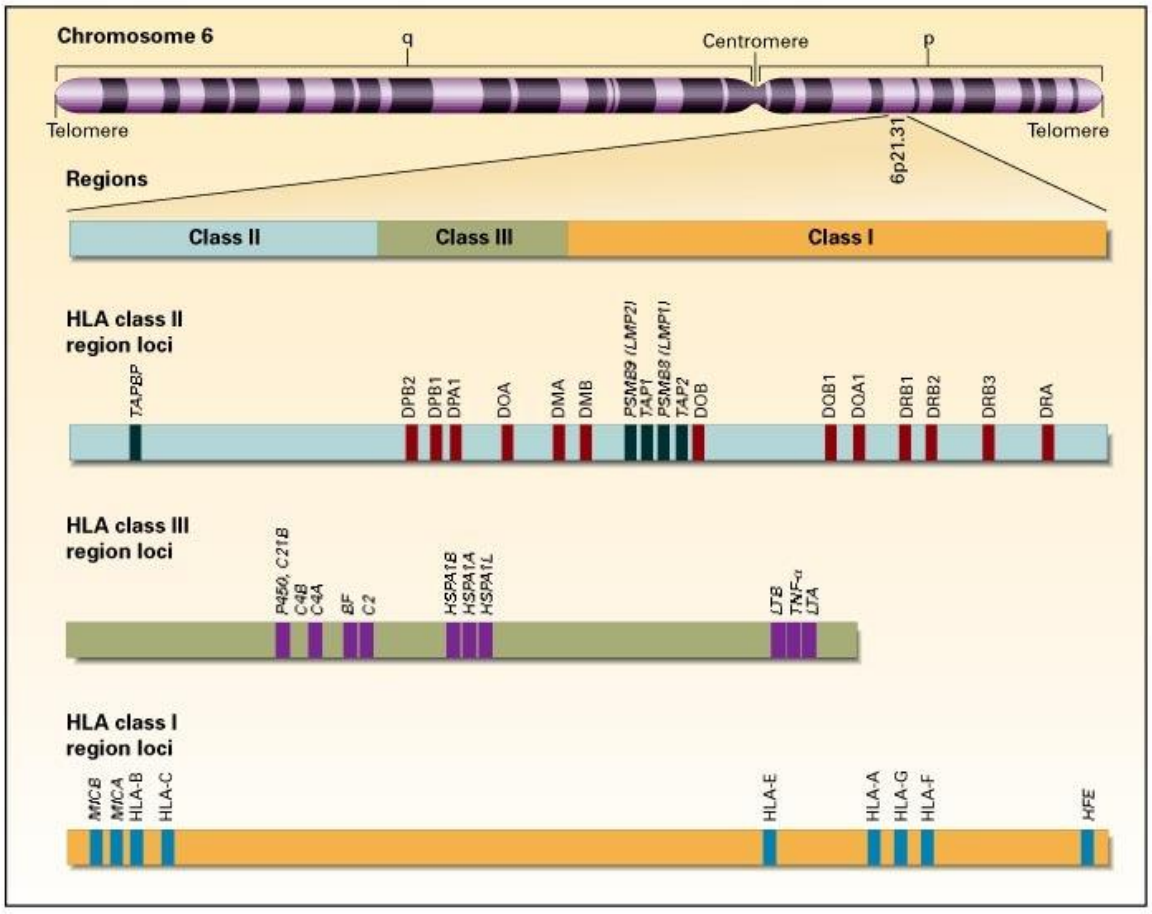


Figure 3 (41) HLA Complex

HLA Class I

The class I HLA molecule has one α polypeptide chain and one β polypeptide chain. Th α polypeptide chain of the class I molecule is coded by class I gene and it has 5 domains; two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the Trans membrane region, and the cytoplasmic tail. The β chain of the class I molecule is encoded by a gene, the beta$_2$-microglobulin gene present on chromosome

15 (Figure 3). There are some 20 class I HLA genes; of which, HLA-A, B, and C, also known as classic, or class Ia genes, are the main players in the immune response. HLA Class I genes are expressed on most of somatic cells, all nucleated cells except mature red cells and the level of expression can vary depending on the tissue (42).

HLA Class II

HLA class II has 2 α and2 β chains. The class II genes code for both types of polypeptide chains of the class II molecules. HLA DR, DQ, and DP are the three heterodimer products HLA Class II genes. There are 4 domains in each of the class II α and β chains: the peptide-binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the trans membrane region, and the cytoplasmic tail. HLA Class II genes are expressed by immune cells like B cells, activated T cells, macrophages, dendritic cells, and thymic epithelial cells (42).However, other types of cells can express class II HLA molecules in the presence of interferon-γ (41).

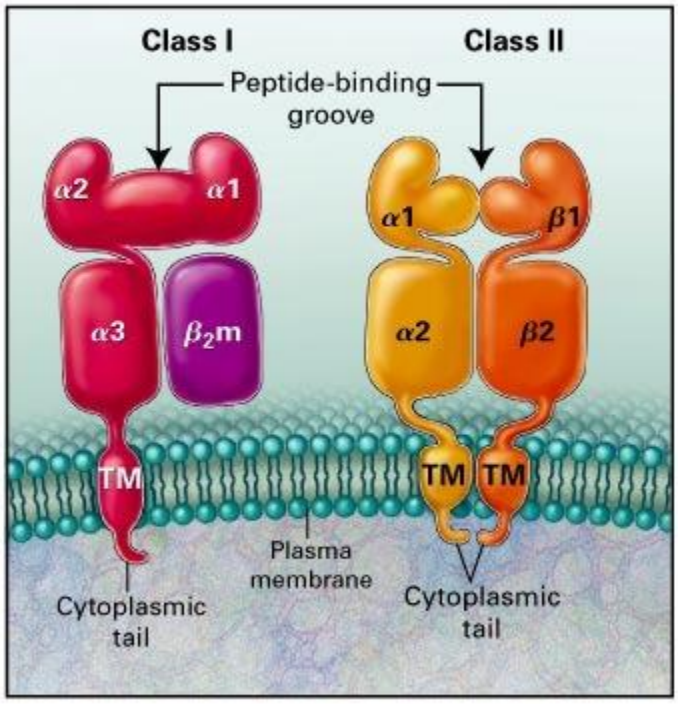


Figure 4 (41) Structure of HLA

HLA SYSTEM AND POLYMORPHISM

The HLA system is highly polymorphic. This polymorphism is mainly clustered in the antigen binding grove and is not uniformly spread throughout the molecule. The peptide binding specificity of HLA molecule is dependent on specific amino acid sequence in the peptide/antigen binding grove and is responsible for the polymorphism of the HLA system. Diversity of HLA polymorphism observed in different ethnic groups can be evolved as adaptation of population in various geographic regions under unique selection pressure. The role of the HLA molecules in presentation of prevalent infectious agents in different areas of the world contributes to ethnic variation in HLA antigens.

FUNCTION OF HLA

The main role of the HLA system is to ensure the integrity of immune system by recognition of 'self' and differentiating from 'non self'. It is the MHC (Major Histocompatibility Complex) of humans. HLA molecules are involved in presenting peptides to the lymphocytes which are involved in protecting our immune system against invaders. The peptides which bind to class I (43) and class II HLA molecules are different in source and nature (44). Only endogenously derived antigenic peptides cell (e.g., cellular, transformed, or virus-induced proteins) will be recognised by Class I restricted T cell receptors [TCR] (CD8+ T cells) and exogenously derived antigens will be recognise by class II restricted T cells receptors (CD4+ T cells) (45).

There are two types of T-cell receptor (TCR) : αβ or γδ (46). Out of which the αβ TCR is present on more than 95% of peripheral blood T cells. Accessory molecules on T lymphocytes are enhancing the interaction between T lymphocytes and HLA molecules during the recognition process between T cell receptor and HLA-peptide complex. The CD4 molecule interacts with a class II HLA molecule on the antigen-presenting cells, and the CD8 molecule interacts with a class I heavy chain on the target cells.

10-30% of peripheral blood lymphocytes lack TCR and exert cytotoxicity without MHC restriction. These cells are called Natural killer cells (NK cells). NK cells can recognize the loss of expression of HLA class I molecules and destroy cells with decreased expression of class I molecules such as some tumours and virally infected cells. When cells with normal MHC class I expression provide appropriate signals to

activating NK cell receptors they can be target of NK cells. NK cells are regulated by activating signal from NK cell receptor – ligand binding and inhibitory signals. There are different NK cell receptors and ligands identified and majority of the ligands are HLA class I molecules (47).

ANTI HLA ANTIBODIES AND RENAL TRNSPLANTAITON

According to the humoral theory of transplantation, antibodies formed against the donor's mismatched antigen with respect to the recipient are responsible for graft rejection. Anti HLA antibodies develop following sensitising events such as transfusion, transplantation and pregnancies. Anti HLA antibodies can be donor specific (DSA) or non-donor specific.

The negative impact of preformed anti HLA antibodies in transplant recipients, on graft outcome was first described by Terasaki et al in 1968 where he crossmatched the serum of 225 potential renal transplant recipients with the lymphocytes of corresponding patient's prospective donor. He observed that the kidney graft failed to function immediately in 24 out of 30 patients with positive crossmatch as opposed to 8 out of 195 with a negative crossmatch($p < 0.001$) (4). From this he suggested that the positive crossmatch in the pre transplant serum against donor lymphocytes can cause graft rejection (4). He had also observed that despite a negative cross match multiparous females or second transplant patients had graft rejection extrapolating the relationship between HLA alloimmunization and sensitising events (4).

After this discovery various platforms have been developed to detect the presence of donor specific HLA antibodies .CDC crossmatch (Microlymphocytotoxicity), FCXM (Flow cytometric crossmatch), ELISA and Luminex based tests are some of them.

Mechanism of action of HLA DSA

The prime targets of antibodies in humoral response in renal transplantation are endothelial cells. Hence humoral rejection can be considered as a form of endothelial dysfunction (43). Kidney has a large endothelial surface area in the glomeruli and in the peritubular capillaries. Endothelium along with underlying smooth muscle and interstitial matrix behaves like a functional unit, integrity of which is very important for regulation of passage of solutes, macromolecules and blood cells to tissues. Endothelial integrity is very important for haemostatic balance as well. The endothelium is coated with heparan sulphate, a proteoglycan which maintains local anticoagulant microenvironment by activating anti-thrombin III. When HLA DSA encounters the HLA antigens in the graft endothelium complement is fixed and complement mediated MAC complex causes breakdown of the graft endothelium .When endothelial integrity is breached sub endothelial tissue factor initiates the coagulation pathway and thrombin is generated. In response to the thrombin, platelets express receptors of vWF and platelets aggregate resulting in micro thrombus formation. These microthrombi cause tissue hypoxia and end organ damage. Another mechanism is antibody dependant cytotoxicity where the antibody binds to the Fc receptors of macrophages and pro inflammatory agents are released causing endothelial damage (48).

In a study by Lachmann, Teraski et al in 2009 as a part of International histocompatibility workshop, 1014 cadaveric renal transplant patients were studied. Of the total, 712 patients did not have pre transplant HLA antibodies detected, and 83% of them had a 5.5-year graft survival. This was significantly higher in comparison to the 93 patients with DSA who had only 49% graft survival over a similar follow up period. They also observed that there were 209 patients who had anti HLA antibodies, but were non donor specific (NDSA). In the latter, the 5.5-year survival was 70% - which is better than patients who had DSA, but lower when compared to patients with no antibodies ($p<0.0001$) This implies that whether donor specific or non-donor specific, anti HLA antibodies cause allograft injury, and therefore in addition to DSA , non-donor specific HLA antibodies can also be considered as biomarkers for long term graft survival(49). When donor specific HLA antibodies are present the negative impact on the graft would be much greater as they are adsorbed on the graft and cause direct injury as well. Such adsorbed donor specific antibodies can cause chronic allograft injury and can be eluted from the rejected graft (50).

In his study Martin et al.eluted out antibodies coating 20 renal allografts which were removed due to chronic allograft nephropathy.19 out of 20 eluates showed anti HLA antibodies. 31.6% of serum samples at the time of transplantectomy and 70.6% of eluates from the nephrectomy samples showed anti HLA DSA, suggesting the role of DSA in chronic allograft nephropathy ($p<0.05$) (50). Adeyi et al. also showed that among patients with renal allograft failure who underwent nephrectomy 11% showed the presence of DSA prior to nephrectomy while 97% showed presence of DSA after

nephrectomy(51). Cardarelli et al studied 251 sera samples from 251 renal transplant recipients at least 6 months post-transplant using ELISA screening tests. Screen positive samples were further tested with ELISA specific panel for antibody specificity. It was found that a 11.2% of patients had anti HLA antibodies and 4.4% of them had donor specific anti-HLA antibodies .He also observed reduced renal allograft survival at 3 year and 5 year post transplantation in presence of HLA DSA(52) . Singh et al studied the pre-transplant sera of 237 deceased-donor kidney transplant patients of high immunological risk. He found that, patients with a class II MFI of 800 detected in single antigen bead flow cytometery tested on the luminex platform, showed an increased hazard ratio for AMR (HR:4.7, p = <0.001) and reduced graft survival (53).

The possible explanation for non-donor specific anti HLA antibodies causing graft failure is that these antibodies can be directed against epitopes which would have been shared with donor's HLA antigens. Essentially here the non DSA behaves as a donor epitope specific antibody, thus causing graft rejection (54–56). In a study by Mao, Terasaki et al where they studied 35 patients with renal transplant allograft failure , 27 patients developed HLA-A and/or -B donor specific antibodies (DSA) and non-donor specific HLA –A and /or –B antibodies(NDSA) while 8 patients developed only non-donor-specific HLA-A and/or -B antibodies(NDSA). 68% of Non DSA detected in patients with both DSA and NDSA showed reactivity against 66 donor-specific epitopes. 39 Non DSA detected in 8 patients showed reactivity to 17 shared epitopes specific to the donor (56).This implies that while non-donor specific anti- HLA

antibodies are less detrimental than DSA, if they show reactivity against shared donor specific epitopes the allograft outcome would be poor.

Everly et al studied DSA of kidney transplant patients who developed acute graft rejection, quantified by luminex SAB at the time of diagnosis of rejection. Out of 52 patients who developed rejection ,31% were detected to have de novo DSA or newly formed DSA(57). Based on the 'Natural history of de novo DSA formation' inflammatory cytokines expressed in the graft immediately after transplantation causes increased HLA antigenic expression. This culminates in B cell allorecognition and de novo DSA formation (58). According to Wiebe C et al de novo DSA can form in 15% lower risk renal transplant patient with no history of pre sensitization and can reduce graft survival at 10 years. His group studied 315 consecutive renal transplant patients without pre transplant DSA, with a mean follow- up of 6.2 ± 2.9 years. A total of 47 out of 315 (15%) patients developed de novo DSA at a mean of 4.6 ± 3.0 years post-transplant. DRB1 mismatch ($p < 0.006$)and non-adherence to immunosuppressants ($p < 0.001$) were independent risk factors for developing de novo DSA (59).

Crossmatching and Methods of detection of HLA antibodies.

In the following section various formats and platforms for anti HLA antibody testing are described

FORMATS OF ANTI-HLA ANTIBODY TESTING

There are 3 formats available for the anti –HLA antibody testing - screening assays, PRA assays and Crossmatching assays

i) Antibody Screening assays:

These are qualitative assays which will detect whether HLA antibodies are present or absent in the sera. It only characterises if the antibodies are to HLA Class I or Class II .Here multiple solubilized HLA antigens are coated onto the solid-phase matrix used either ELISA plates / Bead based Flow cytometry/ Luminex beads. Following incubation of serum with the antigens, a positive reaction indicates the presence of anti HLA antibodies. A negative reaction indicates the absence of anti HLA antibodies.

ii) PRA Assays (Panel Reactive Antibody assay)

The PRA is indicative of how extensively the patient is alloimmunised . Using panels of cells / recombinant antigens representing a wide selection of HLA alleles in varying combinations, the reactivity of patients' serum against HLA alleles is assessed. The test can be performed on any of the platforms inclusive of CDC. The test is reported as "Percent positivity". The higher the percent on a PRA, the lesser the likelihood of finding a compatible donors. The value of PRA depends both upon the composition of the cell panel and the technique used for antibody detection. The composition of the cell panel varies considerably with the use of different commercially available kits or locally procured cell panels. PRA will reflect overall degree of reactivity of the serum as a predictor of immunological compatibility. If a patient's serum reacts with 80 out

of 100 cells in the panel, the patient has a PRA of 80%. That means the patient has a 8 out of 10 chance of acute rejection with a donor graft from that donor pool, and has to wait for a long period until a compatible donor is available. PRA can be assessed using serological (CDC) or solid phase assays such as ELISA or flowcytometry or Luminex beads.

iii) Crossmatching Assays

The presence of preformed anti HLA antibodies in the serum of the recipient can be detected by crossmatching it with donor lymphocytes in CDC crossmatch and Flowcytometric crossmatch. Donor cell lysate can be used for Luminex donor specific crossmatch (XmDSA). Luminex SAB assay with donor's HLA typing is essentially used for Luminex virtual crossmatching.

Various platforms available for testing anti HLA antibodies are

1. Complement dependent cytoxicity / microlymphocytotoxicity
2. ELISA
3. Flowcytometry
4. Luminex based tests

1. Complement Dependent Cytotoxicity (CDC/ Microlymphocytotoxicity)

This is the gold standard test for detection of preformed lymphocytotoxic antibodies and was originally devised by Dr.Terasaki. It is a serological method based on the principle that lymphocytotoxic antibodies in the presence of complement cause cell

death, and the results are visualised by phase contrast microscopy after addition of a vital dye (4). Results are interpreted based on the percentage of dead cells in each dilution with comparison to controls. CDC can be used for donor-recipient crossmatch and Panel Reactive Antibody evaluation. Correlation between a positive CDC crossmatch and renal allograft outcome is well established (60). CDC crossmatch positivity is associated very strongly with hyper acute rejection. Though it is a very cost effective gold standard test in potential donor recipient HLA compatibility testing, it has few disadvantages which can be overcome with other testing platforms:

- CDC cross match requires viable donor lymphocytes.
- It cannot detect the presence of non-complement fixing antibodies.
- It can miss the presence low titre antibodies due to low sensitivity.
- Differentiation between HLA specific and non-specific antibodies is difficult.
- Limited sensitivity and specificity (61)

2. ELISA (Enzyme Linked Immunosorbent Assay)

In ELISA highly purified human HLA class I and II glycoproteins are coated differentially on microtiter plate. If the test sample contains specific antibodies against HLA class I or class II, they will bind to the antigens coated in the wells of the microtitre plate forming antigen antibody complex. This complex is detected using a specific enzyme-labelled (alkaline phosphatase) secondary antibody which is directed against human IgG (conjugate). A chromogenic substrate specific to the enzyme is added to demonstrate the presence of bound antibodies which results in a coloured

product. The reaction is interpreted ELISA reader. ELISA can be used in 3 formats for the detection of anti-HLA antibodies.

i) Antibody Screening

ii) PRA testing

iii) Crossmatching.

3. FLOW CYTOMETRY

It is the simultaneous measurement of multiple characteristics of a single cell as the cell suspension flows in a single file through a measuring device. Flow cytometry measures and characterises physicochemical properties of each cell in a cell population such as size, fluorescence and internal complexity through forward and side scatter. Various fluorescent dyes are used in flowcytomtery which bind or intercalate with different cellular components such nucleic acid or cell membrane. Each cell can be labelled with fluorescent dyes conjugated with antibodies which bind to specific proteins on cell membranes or inside cells. When such labelled cells are passed through the flowcytomter, the fluorescent molecules are excited to a higher energy state by the light source. As fluorochromes return from excited state to their resting state the difference in energy is emitted as light energy at higher wavelengths producing specific colour .By using multiple fluorochrome with similar excitation wavelength and different emission wavelengths producing different colours various properties of a cell can be analysed simultaneously. Commonly used fluorescent dyes include phycoerythrin, propidium iodide and fluorescein.

Flowcytomtery can be used in 3 formats in the detection of anti-HLA antibodies

i) Antibody Screening

ii) PRA testing

iii) Flowcytometric crossmatching.

In flow cytometric crossmatch donor lymphocytes are incubated with recipient serum to allow donor specific anti HLA antibodies to attach to the lymphocytes. This antigen antibody interaction is further detected by flow cytometry using a conjugate of antihuman globulin with a fluorescent dye. T cells and B cells are gated using CD 3 and CD 19 fluorescent conjugates respectively and analysed separately

4. Luminex Technology.

The invention of polymerase chain reaction by Dr. Kary Mullis was a Nobel Prize winning breakthrough of 20th century. With the advent of this technology, the landscape of clinical histocompatibility testing has significantly changed. The DNA based tests revealed the molecular complexity of HLA system that was not identified with the previously available serological assays. The number of HLA alleles had significantly expanded from hundreds to several thousand, necessitating the advent of assays to type them with accuracy and precision. This necessity led to the development of microbead based Luminex technology in 1990s for HLA typing (62). Soon, the same multiplex bead technology was started for the detection of anti HLA antibodies.

In Luminex multiplex bead technology, purified HLA class I and class II molecules are coated on color-coded polystyrene beads. These beads are labelled with different ratios of two fluorescent dyes identifying discrete bead populations that bear distinct HLA antigens. A fluorochrome tagged anti-human -IgG (secondary antibody) is used to assess the binding of HLA. Data output is reported as mean fluorescence intensity (MFI) which is a semi quantitative parameter reflecting the strength of anti HLA antibody.

Luminex beads can be used for screening, PRA estimation as well as for virtual crossmatch

a)Mixed antigen screening Beads.

Here each bead carries a mixture of purified HLA class I and II molecules from three or more donors(63) . In the screening assay beads are incubated with patient's serum where antibodies bind to solubilised antigens on the beads. Thereafter fluorochrome tagged anti-human IgG (secondary antibody) is added that will bind to HLA antigen antibody complex. The resultant fluorescent shift in the test sample in comparison to negative control, provides a qualitative assessment of the presence or absence of anti HLA antibodies..

b) Phenotype/Single Donor/PRAbeads

The ratio of expression of HLA antigens on these beads is similar to that observed on the actual cell surface and therefore these beads give a closer "physiologic representation of actual cells" with the added sensitivity and specificity inherent of the luminex assay. The phenotype beads, or single donor beads, provide 'cell-like' reagents by virtue of having six HLA class I or class II antigens of a single donor and are used for PRA estimation. It will give the result as a PRA%. It may not always be sufficient for accurate and complete assignment of HLA-antibody specificities in patients who are highly sensitized.

c) Luminex Crossmatches

It can be Luminex virtual crossmatch or donor specific crossmatch (XmDSA)

i) Luminex Virtual Crossmatch (vXM)

The precise assessment of anti HLA antibodies present in sera of sensitized patients in conjunction with objective identification of the HLA antigens expressed by the potential organ donors is defined as virtual crossmatch (vXM). Luminex SAB assay with donor's HLA typing is essentially used for virtual crossmatching.

Luminex Single antigen beads (SAB)

In this assay, each polystyrene micro bead is coated with multiple copies of single HLA allele and therefore it is known as Single antigen bead. Out of a set of 100 beads, each bead is having a unique dye combination to give a fluorescent coding system. As beads are incubated with recipient's serum, anti-HLA antibodies will bind to their

specific HLA target and this can be identified by fluorescence emitted from fluorochrome labelled secondary antibody while passing through the flow analyser. Data output is reported as mean fluorescence intensity (MFI) reflecting the strength of antibody

ii) Luminex Donor specific crossmatch (XmDSA)

In this assay donor specific HLA antigens from donor lymphocyte lysate are adsorbed to the capture beads. When these capture beads are incubated with patient's serum, the antibodies bind to their specific HLA target indicated by fluorescence from fluorochrome labelled secondary antibody. Here also data output is reported as MFI and it differentiates between Class I and Class II antibodies.

In comparison to the Luminex SAB, this assay has following disadvantages

- It is standardised for detection of antibodies to the HLA A, B, and DR loci, and not for antibodies to the HLA C, DP and DQ loci.
- It can give false positivity for HLA class II antibodies especially in the presence of high levels of immunoglobulins in the sera (61).

NON HLA antibodies in transplantation

Though HLA molecules on the graft are the main histocompatibility targets of recipient's immune response in eliciting cellular or humoral rejection, increasing number of non HLA antibodies have been identified over the last decade. There are increasing number of cases with renal allograft dysfunction or rejection episodes in

patients who are not known to have any donor specific or non-donor specific anti-HLA antibodies.

In his study Opelz et al raised the possibility of non-HLA immunity contributing to the long-term renal allograft outcome. A total of 4000 HLA identical renal transplant recipients having varying PRA were followed up for 10 years for graft survival. 72.4% of patients with no PRA had a 10 year graft survival vs. 63.3% survival in patients with 1-50% PRA ($p=0.0006$) and 55.5% survival in patients with PRA>50% ($p<0.0001$). As all these transplant recipients were HLA matched, it raised the possibility that high PRA detected by lymphocytotoxicity would be an indicator of heightened immunity against non HLA transplantation antigens. He also suggested that the targets for antibodies causing late renal allograft rejections could be called minor histocompatibility antigens. (64). Later Angaswami et al has reviewed the role of non-HLA self-antigens in eliciting immune response and negatively affecting the allograft outcome (65) .

Non-HLA antibodies can be either autoantibodies or alloantibodies and can be present prior to transplantation or can be formed de novo post transplantation. They are not always directed against the targets on endothelium. Li et al had tested pre- and post-transplant samples of paediatric renal transplant patients in protoarray for reactivity against over 5000 protein targets selected on appearance in the kidney. It was observed to have positive reactivity against 61% of the targets on average. The highest reactivity was against Stathmin-like 3 antigens expressed in the cortex and renal pelvis(66) In another study by Porcheray et al. it was observed that antibodies produced by B-cell clones are polyreactive instead of directed against a single antigen-

target. As they isolated B-cell clones from a recipient with AMR it was interesting to find antibodies produced from some of these B cell clones were reactive not only to HLA and MICA, but also to auto-antigens including DNA and cell cytoplasmic structures (67)

For non HLA autoantibodies to form there should be a gradual loss of self-tolerance. Various mechanisms are postulated for developing auto antibodies.

- When there is inflammation there would up regulated antigenic expression, altered antigenic processing and expression of cryptic epitopes. Antibodies will be produced against these antigens(68,69).
- Inflammation induced oxidative stress, post translational modification and apoptosis cause neoantigen against which antibodies are produced (70).
- Cross reactivation of auto reactive B and T cells due to the close resemblance of infectious agent and self-peptides (68,71,72).
- Auto reactive B cell clone is usually prevented by follicular T helper cells (Tfh). When there is defective Tfh through impaired B cell selection autoantibodies are produced (73,74).
- In response to the renal allograft or donor HLA antigens there will be increased Th17 generation which causes impaired deletion of immature auto reactive B cells (73).

The non HLA antibodies studied at least in two cohort studies so far are

- Anti-endothelial cell antibodies (AECA) (75)
- Angiotensin II type 1 receptor antibodies (AT1R antibodies) (76)

- Endothelin-1 type A receptor antibodies (anti- ETAR antibodies) (77)
- MHC class I related chain A antibodies (MICA) (78)

Other non HLA antibodies studied in case control studies are

- Anti Vimentin (79)
- Anti LG3 (80,81)
- Anti-apoptotic antibodies(82)

Among all these antibodies Anti-endothelial cell antibodies (AECA) and MHC class I related chain A antibodies (MICA) are allo antibodies. Angiotensin II type 1 receptor antibodies (AT1R antibodies), Endothelin-1 type A receptor antibodies (anti- ETAR antibodies). Anti Vimentin, Anti LG3 and Anti-apoptotic antibodies are autoantibodies.

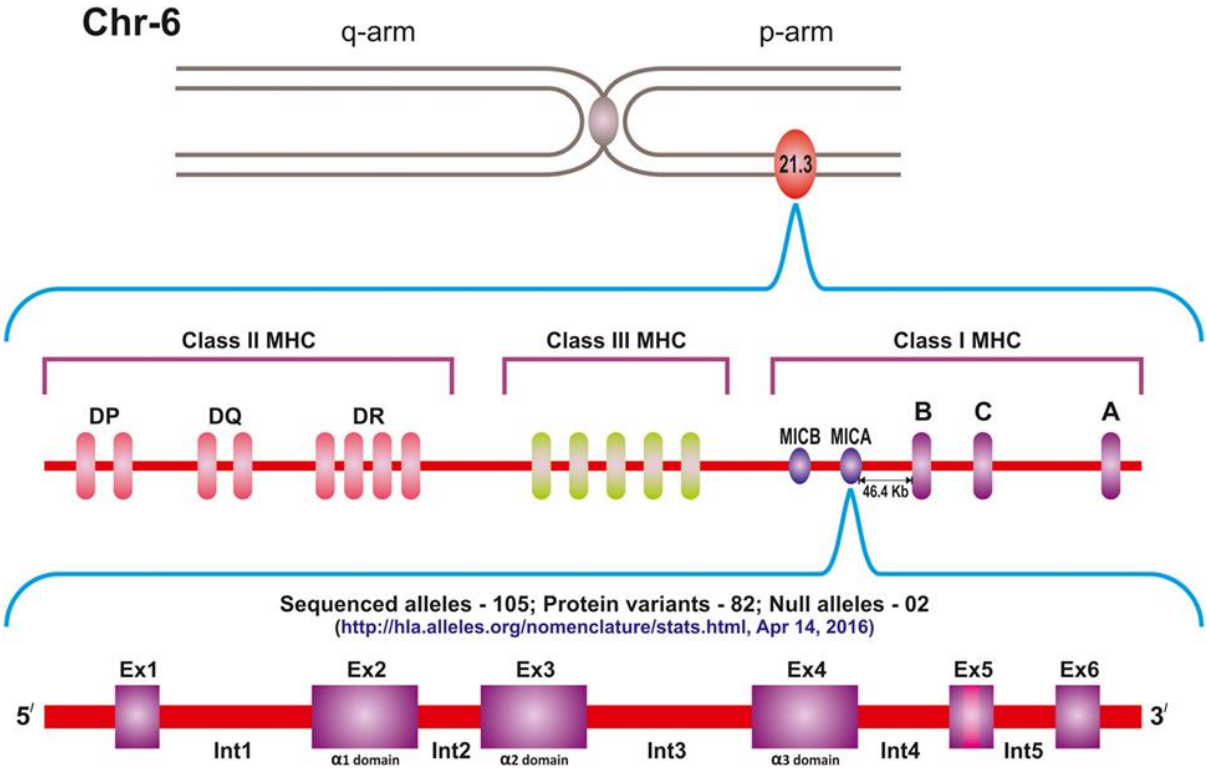
MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I CHAIN-RELATED ANTIGEN A (MICA)

The Major Histocompatibility Complex (MHC) class I chain-related genes A and B (MICA and MICB) are a family of proteins first described in by two independent groups of researchers in 1994. These proteins are encoded in HLA Class I genes. First group from Perth referred to them as Perth beta block transcript 11. Bahram and co-workers from Harvard named them as MIC, which was later adopted by the World Health Organization nomenclature committee for factors of the HLA system (83,84). MICA antigens are expressed on the surface of endothelial cells, keratinocytes, monocytes, and dendritic cells.

STRUCTURE

MHC Class I chain related (MIC) genes are located within the MHC class I region of chromosome 6 p21.3. A total of seven MIC genes, designated as MICA to MICG have so far been identified. Of which the only functional genes are MICA an MICB while MICC to MICG are pseudo genes (85,86). MICA gene is in very strong linkage disequilibrium with HLA- B locus as it is located centromeric to HLA-B locus at a distance of 46.4 kb.

Figure 5 (6) **Location of MICA gene**



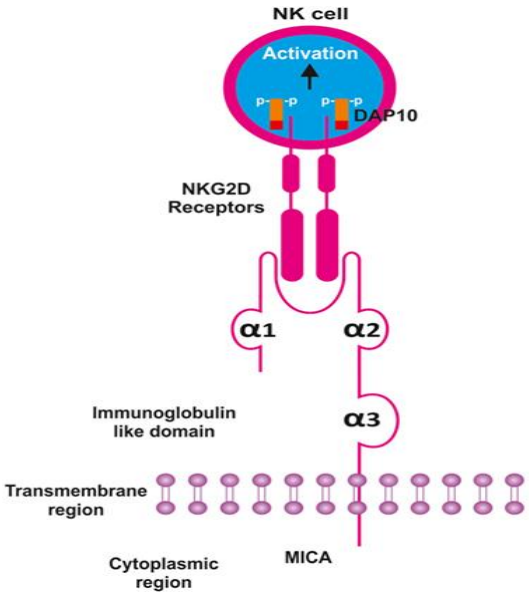
MICA is structurally similar to classical HLA class I molecules with 30% sequence homology and three extracellular domains. The α1 domain is encoded by exon 2, α2 by exon 3, and α3 by exon 4. Exon 5 codes for trans membrane (TM) region, while

the carboxy-terminal cytoplasmic tail is encoded by exon 6. There are five introns of which the largest intron is the first one. The gene has 11.7 kb size and is transcribed into an mRNA of 1,382 bp, which gives rise to 383-amino acid polypeptides of 43 kDa including the leader peptide (6).

Unlike the HLA class I molecules, the MICA does not have β_2-microglobulin (β_2-m). Though the domain structure of MICA looks very similar to its classical class I counterpart, its α-2 helix is disordered and flexible making it unsuitable for peptide binding. Unlike HLA class I molecules, the platform formed by the α1 and α2 regions of the MICA molecule points downwards toward the cell membrane thus exposing its underside to the intercellular space. However, as MICA interacts with its receptor NKG2D, the flexible α2 helix becomes ordered by a further two alpha-helical turn and the α1 and α2 domains flip back 96°.

FUNCTION OF MICA

Functionally MICA is different from classical HLA molecules and is not involved in antigen presentation to T cells. Instead they act as ligands for the activating natural killer (NK) group 2, member D (NKG2D) receptor which is expressed on NK cells, γδ T cells, and CD8+ αβ T cells. Interaction of MICA with NKG2D leads to NK cell responses, cytokine production and activation of antigen-specific cytotoxic T-lymphocyte-mediated cytotoxicity (87). Polymorphic MICA antigens are capable of inducing antibodies that can kill target cells in the presence of complement (88). Hence MICA plays a key role in linking the innate and adaptive immune responses in organ transplantation.



Structure of MICA FIGURE 6 (6)

POLYMORPHISM OF MICA

MICA is a highly polymorphic non-classical class I gene with 105 already reported alleles and new alleles are being continuously identified (83). This polymorphism is different from that of the HLA genes in various aspects.

- The magnitude of polymorphism is far less than that seen in the HLA system.
- In contrast to the HLA class I molecules, where the polymorphism is located in the proximity of antigen binding groove, the MICA polymorphism is dispersed to all the three extracellular domains with the greatest variability in the α2 domain, encoded by exon 3.
- observed variations in the TM region for several MICA alleles despite having identical extracellular domains also contribute to the polymorphism.(89)

Though MICA is highly polymorphic, MIC B is less polymorphic and only 45 alleles are reported so far. There is no robust evidence to support its role in transplant outcome (84).

Mechanism of production of anti-MICA antibodies

MICA antigens are expressed on the surface of endothelial cells, keratinocytes, monocytes, dendritic cells and not on peripheral lymphocytes.. Sensitisation via pregnancy and transplantation are known to cause anti MICA antibody production (90). However the role of transfusion in this aspect is still inconclusive (91)(92).. (93).There would be an enhanced expression of MICA antigens on the allograft due to the physiological stress associated with transplantation.: It was shown that an A5.1 mutation in the donor, which is related to the MICA*008 allele, is associated with higher MICA expression in donor endothelial cells compared to wild type donors and therefor these mutated MICA proteins are important targets for antibody formation (94). Furthermore, mismatching on certain amino acid residues leads to increased MICA antibody formation (95)Various factors enhancing the expression of MICA antigens described are stress conditions such as ischemia reperfusion injury (96), autoimmune diseases (97), cytomegalovirus (CMV) infections (98) and tumoral transformation (99). So it can be considered as stress biological marker which can induce antibody production (100).

Immune response to MICA

The polymorphic nature of MICA antigenic system is the basis of immune response to foreign MICA antigens. This was first described by Zwirner et al where he has observed presence of anti-MICA antibodies in the sera of solid organ transplant recipients (90). Similar antibodies were reported in mice immunized with recombinant MICA by Zou et al (88). In 2007, the landmark study by Zhang and Stastny demonstrated that immunization of mice with recombinant MICA*001 having all the three extracellular domains can stimulate T cell proliferation and cause cell mediated cytotoxicity (101). In the same study it was described as through various mechanisms MICA antigens are capable of eliciting immune response and are rather immunogenic. This immunogenicity can be due to cross reactivity with unidentified microorganisms, genetic factors or expansion of responding immune cell repertoire.

ANTI-MICA ANTIBODIES:

The impact of anti-MICA antibodies on organ transplantation is described under following headings.

1. Impact in solid organ transplantation

2. Impact in renal transplantation.

1.Impact in solid organ transplantation:

The MICA antigenic system has been investigated by many groups as a potential target impacting graft rejection and dysfunction in solid organ transplantation

.Zwirner et al has described that sera from 56 kidney allograft recipients and 17 recipients of transplants of other organs with either early rejection, high PRA, or presence of antibodies against keratinocytes reacted with different recombinant MICA molecules (90).

Using human alloantibodies and mouse monoclonal antibodies Zou et al has demonstrated MICA antigens as the target of complement dependant cytotoxicity (88). In 2002 many groups demonstrated that anti –MICA antibodies could have an important role in precipitating antibody mediated rejection in solid organ transplantation. . Hankey al demonstrated the MICA antigenic expression on renal and pancreatic allografts (102). In the same year Sumitran – Holgersson et al has described significant correlation between anti-MICA antibodies and renal allograft loss. Their group investigated 139 pre-transplantation serum samples for anti-MICA antibodies .37 out of 139 samples were positive for anti-MICA. 81.1% of those who had anti-MICA positivity developed rejection in contrast to 38.2% with MICA negativity who experienced acute graft rejection ($p<0.001$) The test checked the specificity of MICA genotyped kidney micro-vascular endothelial cell (KMEC)-reactive antibodies using MICA transfected cells and microlymphocytotoxicity (7).These studies along with the widespread availability of bead based luminex technology opened doors for more studies on the role of anti-MICA antibodies in solid organ transplantation. So from the year of 2007 onwards many studies were published on impact of anti-MICA antibodies and solid organ transplantation especially in Kidney, heart and liver transplantation. A number studies have described cardiac allograft rejection episodes and correlation with anti-MICA antibodies (103). There are limited number of studies

on the impact of anti-MICA antibodies in liver transplantation (104). Those studies could not find any association with graft rejection or graft survival with anti-MICA antibodies as there is limited expression of MICA antigens on hepatocytes.

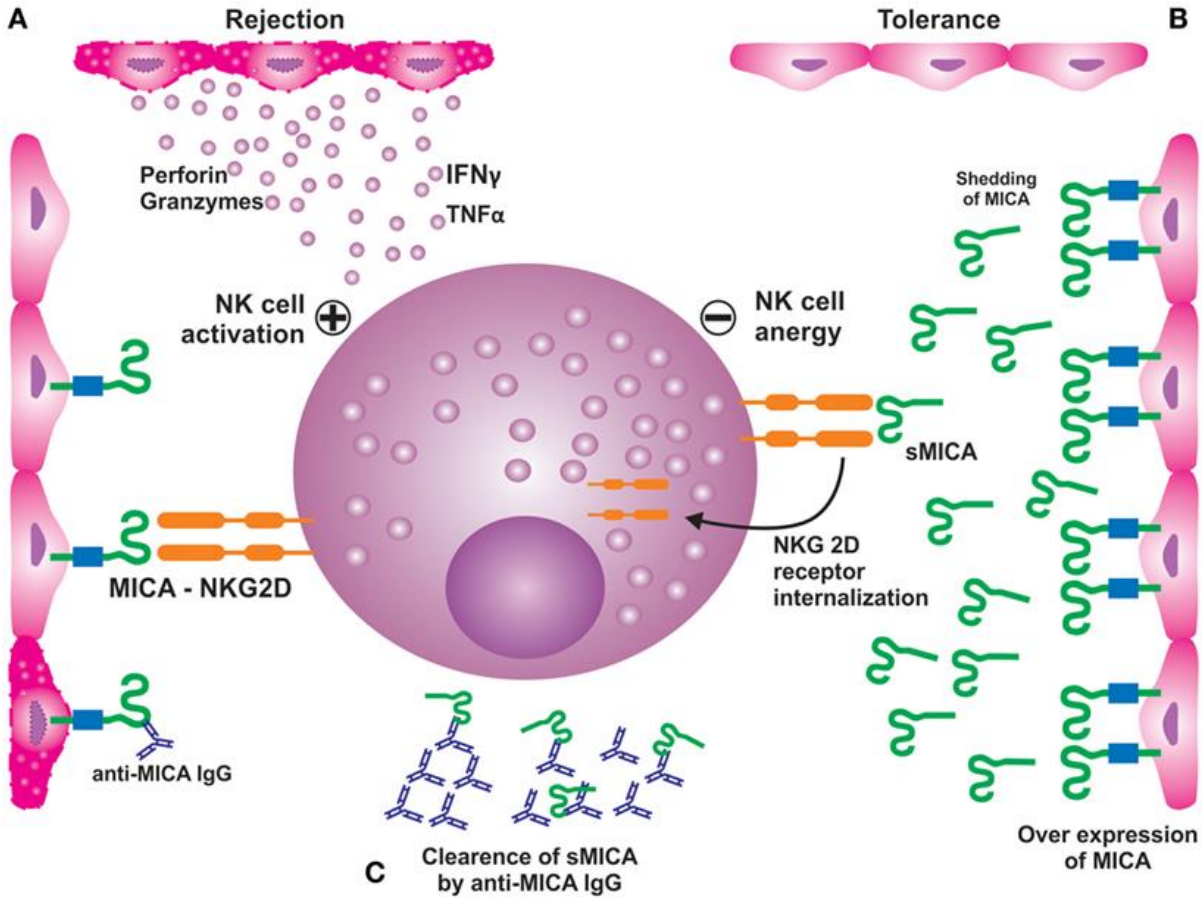
Soluble MICA and solid organ transplantation

In addition to the endothelial membrane bound MICA a soluble isoform of MICA (s MICA) exists which is implicated in the suppression or impairment of NKG2D-mediated immune response. sMICA is formed by over expression of MICA on endothelial cells membrane and its proteolytic shedding from the surface. They interact with the NKG2D receptor, resulting in its internalization and degradation of receptor–ligand complex and thus suppress NKG2D-mediated host innate immunity.

Several studies have shown the role of sMICA in allograft tolerance in solid organ transplantation. Suarez-Alvarez et al, (105) demonstrated an inverse relationship between sMICA levels and acute rejection while evaluating the role of MICA on heart transplantation. In their study on 31 heart transplant recipients with a follow-up of 1 year, 8 patients suffered acute rejection while the remaining 23 patients did not develop any rejection episodes. On further analysis it was found that 17 out of 23 patients without rejection had detectable levels of sMICA as compared to two patients who rejected the graft ($p < 0.03$). In the same study it was observed that those patients having no sMICA had tendency to produce anti MICA antibodies leading to transplant rejection. On the other hand the sMICA levels were detected in patients with no MICA antibodies in absence of rejection. The same authors in another study (103) have monitored sMICA levels in 34 heart transplant patients in pre transplant serum

samples and at 15 days, 3 months and 1 year post transplantation samples. sMICA was not detected in the pre transplant sera, while it was detected in 21 patients at 15 days post transplantation. 20 of these 21 patients did not develop any rejection episode (p = 0.0001). 9 of the 13 patients who had no detected levels of sMICA, developed acute rejection. These observations suggest that presence of sMICA contributes to better graft acceptance.

Figure 7 (6) Antagonistic effect of sMICA vs. anti-MICA antibodies in solid organ transplantation



2. Impact in Renal transplantation

The impact of anti-MICA antibody has been studied more extensively in renal transplantation in comparison to other solid organ transplantation. The possible mechanisms involved in renal allograft rejection by anti-MICA antibodies include

- Complement dependent cytotoxicity : According to Zapardiel et al., preformed anti-MICA antibodies in the serum of transplant recipients can bind native MICA antigens on cell surfaces and activate the classical complement cascade leading to target-cell death (93).
- NKG2D-mediated cytotoxicity. Anti-MICA antibodies result in clearance of soluble MICA antigens which are implicated in suppression of NK cell mediated cytotoxicity. sMICA interact with the NKG2D receptor, resulting in its internalization and degradation of receptor–ligand complex and thus suppress NKG2D-mediated host innate immunity(Figure 6).

After Zwirner's study many studies have shown a relationship between pre- or post-transplant anti-MICA antibodies and kidney allograft loss (7,91), dysfunction, and/or acute rejection (95).There are several serial long-term follow-ups reporting an association of anti-MICA antibodies with chronic graft failure (78,106). Anti-MICA antibodies have been extensively investigated in over 35 renal transplant studies so far (4).

In 2002 Sumitran et al., had investigated 139 pre-transplantation serum samples for anti-MICA antibodies and 37 out of 139 samples were positive for anti-MICA. 81.1%

of those who had anti-MICA positivity developed rejection in contrast to 38.2% with MICA negativity who experienced acute graft rejection ($p<0.001$) (7).

Following this the next major study on the impact of anti-MICA antibodies on renal allograft outcome was an international collaborative study conducted in 20 centres across 13 different countries, by Zou et al.(91). In that study 1910 renal cadaveric allograft recipients were tested for anti-MICA antibodies in pre transplantation serum samples using the Luminex platform. 11.4% of patients were found to have anti-MICA antibodies. Among the recipients with anti-MICA antibody positivity 1 year mean graft survival rate was 88.3±2.2% compared to 93.0±0.6% among those without anti-MICA antibodies ($P = 0.01$).The survival rate was even lower among MICA antibody–positive first time kidney transplant patients (87.8±2.4%) than among MICA antibody–negative first time renal allograft recipients (93.5±0.6%, $P = 0.005$). The graft loss associated with anti-MICA antibody was seen mostly in the immediate post transplantation period. The reduced allograft survival associated with anti-MICA antibody was more evident in kidney transplant recipients with good HLA matching underscoring its high immunogenicity and clinical significance. It was also noted in the study that blood transfusion was not significantly associated with anti-MICA antibody production in contrast to previous studies.

The next major study was published by Lemy et al in 2010 (92) in which 494 healthy controls and 597 patients with chronic kidney disease (CKD) were tested for anti-MICA antibodies using the Luminex platform (LSA-MICA) in pre transplantation

serum Out of 597 patients 425 patients had already undergone transplantation and their pretransplant sera was used. Remaining 172 patients were CKD patients undergoing regular haemodialysis. 23 MICA specificities were assayed in comparison to 5 in study by Zou et al. Prevalence of MICA antibody in the whole cohort was analysed and it was found that 14.9% of patients and 4.7% of healthy controls showed positivity for anti-MICA antibodies. In the same study anti-MICA antibody was observed to be significantly associated with blood transfusions, previous organ transplantation and parity with two or more pregnancies. It was also noted that 'Uraemia' per se was associated with anti-MICA antibody formation. This could be possibly due to the enhanced MICA antigenic expression secondary to uraemia induced endothelial stress and contact with dialysate fluids and filter (107). It was intriguing that one third of patients with CKD were positive for anti-MICA antibodies and had no sensitising events implicating other possible mechanisms for MICA sensitization. In addition to that 20% of CKD patients were detected to have anti-MICA antibodies that were auto-reactive. Although there were frequent acute rejection episodes with features of AMR among patients with anti-MICA antibody positivity (5 out of 6 as opposed to 19 out of 47,p=0.08) one year graft survival was similar in anti-MICA antibody positive (94.9%) and negative(94.1%) groups. Graft survival was followed up for 10 years. Lemy's group found better 10 year over all survival in patents with anti-MICA antibody positivity than patients who were tested negative for anti-MICA antibodies. 10 year overall survival was 88% for patients who were positive for anti-MICA antibody as opposed to 74% in patients who were negative which was statistically not significant (88% vs. 74%, P= 0.25)(92). The authors discuss this as possibly

attributable to the significantly higher degree of immunosuppression that their patients were treated with, thus nullifying the impact of anti-MICA antibodies detected (92).

In 2013 Sanchez –Zapardiel et al reported prevalence of anti-MICA antibodies in patients waiting for renal transplantation as 7.15% in a retrospective study on 727 transplant patients using Luminex platform (LSA-MICA). These preformed antibodies were not related to previous transplantation, sensitising events or anti HLA antibodies. Preformed anti MICA antibody was reported as a risk factor early post-transplant allograft rejection. However there were no significant epidemiological or clinical differences observed between anti-MICA antibody positive and negative study groups. Allograft survival and rejection rates at 2 years post transplantation was also not significantly different between these 2 groups. Donor specificity of anti-MICA antibodies was not examined in that study(108).

In 2016 the same authors further demonstrated that preformed anti-MICA antibodies can bind native MICA molecules on the cell membrane. By using both the C1q single-antigen beads (SAB) assay and complement dependent cytotoxicity (CDC) they have demonstrated anti MICA antibodies mediate cell death by fixing and activating the complement cascade (93).

The role of donor specific anti MICA antibodies in both type II Banff acute cellular rejection (ACR) and antibody mediated rejection (AMR) was described for the first time by Narayan et al in a highly sensitized paediatric renal transplant patient (109). Anti-MICA DSA of >15000 MFI in pre and post-transplant serum of patient was associated with acute rejection. The authors also underscored the significance of

detection of donor specific anti MICA antibodies and serial monitoring of the strength of antibodies (MFI) especially in highly sensitized renal transplant patients.

According to cox et al a correlation was observed between anti MICA antibodies and ACR. Among a cohort of 442 renal transplant patients 391 patients underwent renal biopsy. Out of these 391 patients 70 patients had ACR. 35% (9/26) of patients with Anti-MICA antibody developed ACR in comparison to 17% (61/365) anti-MICA antibody negative patients.[multivariate OR=1.2, univariate p=0.021]. The presence of both anti HLA and anti-MICA antibody showed significant association with ACR with a calculated Odd's ratio of 4.9 and p value of 0.018(95).

In 2013 Mehra et al has described a case report where preformed donor specific anti MICA antibodies of higher MFI (10,000- 20,000) was implicated in hyper acute to acute renal allograft rejection (110).

In another study by Ming et al describes donor specific MICA antibody with MFI >10,000 as a risk factor for acute antibody mediated rejection (111) especially for re transplants. Using HUVEC culture cells this group demonstrated the cytotoxic potential of anti-MICA antibody via complement fixation.

A recently published study by Chowdhry et al showed 14.6% samples (94 out of 646) were positive for anti-MICA antibodies for one or more MICA allele, using LSA MICA. 35 patients out of the 94 patients had anti MICA antibody with single allele specificity. The average MFI in this group wad 2584. 59 out of 94 patients had shown anti-MICA antibody positivity to multiple alleles and the average MFI in this group

was higher (4396) than that of the group with single allele specificity. On comparison of graft survival between patients with no anti-MICA antibody versus patients with single/multiple anti -MICA antibody/antibodies it was found to that anti-MICA antibody positivity was associated with reduced graft survival ($p<0.05$). Patients who were tested positive for anti-MICA antibody had 89.3% graft survival as compared to 94.7% in patients who were tested negative for anti-MICA antibody ($P<0.05$). 12.3% out of 646 renal pre transplant patients with isolated anti-MICA antibody positivity without any anti-HLA antibodies ,developed poor graft outcome.((hazard ratio of 2.768, $P<0.05$) . The most common antibody identified among patients with anti-MICA antibody was antiMICA∗041(35.8%) (112)

In a case control study by Mizutani and Terasaki et al 63 cases of renal allograft rejection and 82 controls with good graft outcome were studied. 52 % cases and 21 % of controls showed anti-MICA antibody positivity ($p=<0.001$). Anti-MICA antibody positivity was tested by CDC using recombinant MICA cell lines. In the same study they have suggested that anti MICA and anti HLA antibodies can independently involve in the process of graft failure (113)

Both studies of Chowdhry et al (112) and Zou et al (91) the co-existence of anti HLA antibodies and anti-MICA antibodies were quite rare and was postulated to be not significant. In his study Chowdhry et al described 15.2% of total patients were positive for anti HLA and anti-MICA antibodies. However the co-existing antibodies did not reduce or affect the graft survival (Hazard's ratio of 1.3750, P>005)(112). Zou et al in their study with 1919 patients observed that only 1.9% had both anti-

MICA and anti HLA class I antibodies and 1.8% had both anti-MICA and anti HLA class II antibodies. They also showed that poor graft outcome correlated with the presence of anti-MICA antibodies in recipients who were not sensitized with HLA(91).This was in contrast to the study by Mizutani et al where he described that patients with both anti HLA and anti-MICA antibodies rejected their grafts more frequently than those who did not have either of these antibodies.77% of patients with failed transplants showed presence of both IgG HLA and anti-MICA antibodies as opposed to 42% of patients with functioning grafts($p<0.01$) (106). The study by Ozawa at al described anti-MICA antibodies positivity in post-transplant sera of 12% of 266 kidney recipients. Almost all patients with anti-MICA antibody positivity showed positivity for anti HLA antibodies also(114) .

There is no current literature available on the significance of NBG ratios of anti-MICA antibody detected. However in the context of anti HLA antibodies and in a stem cell transplant setting, a study by Delbos et al (115) testing the significance of anti HLA antibodies on the occurrence of GVHD found an association of GVHD with an NBG ratio >3 determined independently by the lab as cut off.

In another study by Endres et al 7920 blood donors were screened for the presence of anti HLA antibodies using LABScreen mixed antigen test. Blood donors were recruited from the “Leucocyte antibody prevalence study”. Results were expressed as NBG ratio. The manufacturer’s recommendation was a cut off value of 2.2 – NBG ratio to determine positivity. However, they also allow the labs to determine their own independent cut offs. This study established cut offs of 10.8 for HLA class I and 6.9 for HLA class II based on standard lab procedures. Using the single antigen bead

assay as a comparator subsequently, these cut off values correlated with 78% and 79% concordance with SAB for HLA class I and Class II antibodies respectively without missing out any true positives. (112)

In a study by Fadei et al (116) 96 apheresis platelet donors were screened for the presence of anti HLA antibodies using LABScreen Mixed assay. Positivity of anti HLA antibody was expressed by the NBG ratio cut offs provided by the manufacturer One Lambda. In the study they concluded that if the mixed antigen assay is to be used for screening blood donors for anti HLA antibodies, due to the significant number of "undetermined" NBG ratios obtained, it would be worthwhile to do a second confirmatory assay to prevent inappropriate exclusion of donors.

RESULTS

Among 88 patients who underwent renal transplantation, there were 44 controls and 44 cases.

1. GENDER CHARACTERISTICS

1.1 Overall Study Group

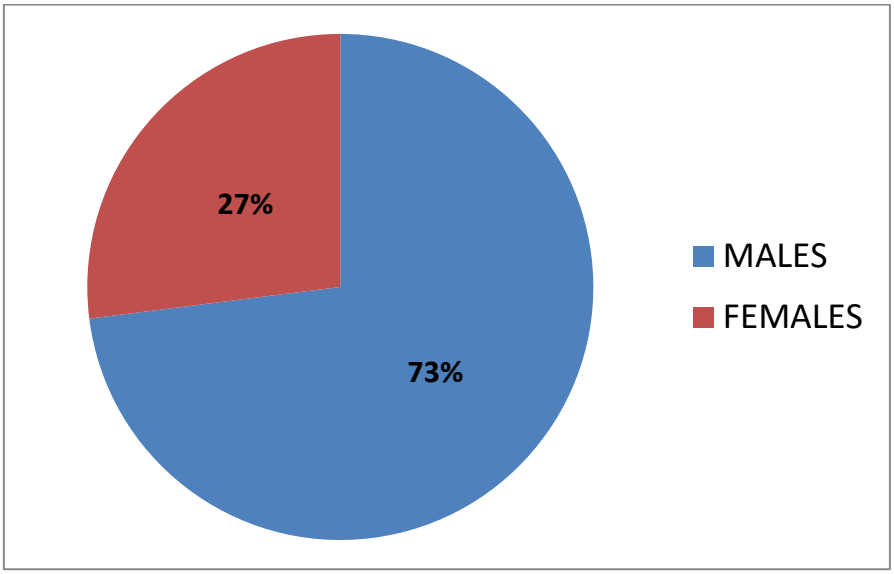


Figure 8: Gender distribution in the overall study group

Out of 88 patients, 64(73%) were males and 24 (27%) were females.

1.2 Cases

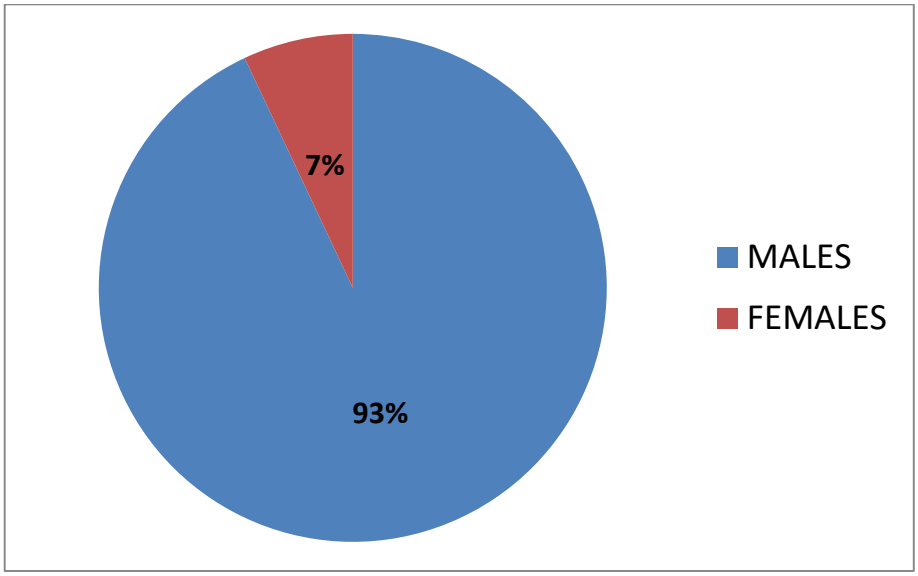


Figure 9: Gender distribution in the CASES

Among cases 41(93%) were males and 3(7%) were females.

1.3 Controls

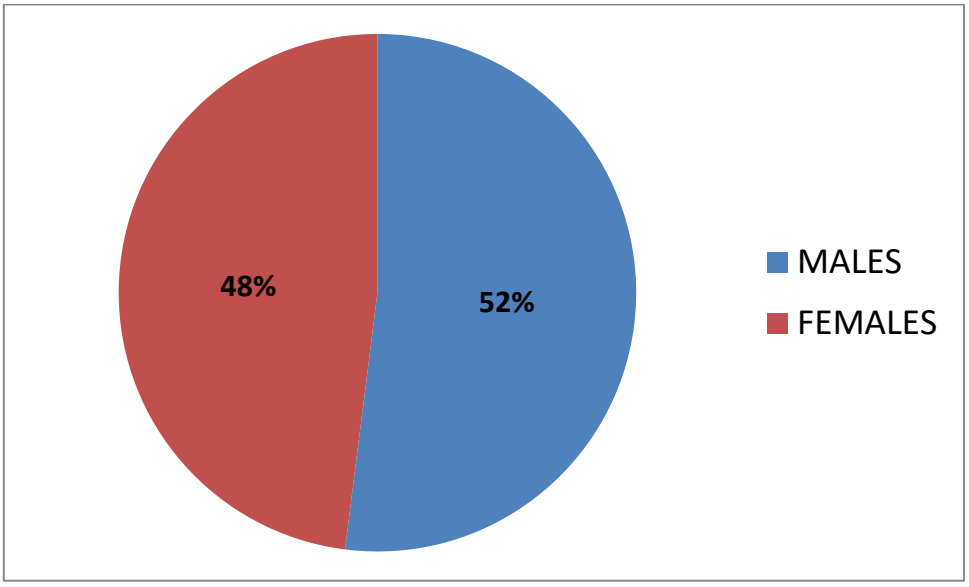


Figure 10: Gender distribution in the Controls

In controls 23(52%) were males and 21(48%) were females.

2. Age Characteristics

Table 4: Age characteristics of the study groups

Group	Mean Age (years)	SD	Range (years)
Overall study group	35.33	10.93	18- 62
Cases	34.14	9.88	18-59
Controls	36.52	11.89	21- 62
Male	35.00	10.28	18- 60
Female	36.20	12.71	21- 62

3. Demographics:

Geographic distribution of the study population has been described in Table 4. As our hospital is a referral centre for patients from rest of India and South East Asia the study population was distributed across various states of India and neighbouring countries like Bangladesh (15.9%), Bhutan (10.2%) and Nepal (2.3 %).

Table 5: Geographic distribution of patient population

STATE	**N**	**%**
Andhra Pradesh	2	2.3
Assam	1	1.1
Bangladesh	14	15.9

Bhutan	9	10.2
Bihar	5	5.7
Chhattisgarh	11	12.5
Jharkhand	12	13.6
Kerala	3	3.4
Madhya Pradesh	1	1.1
Meghalaya	1	1.1
Mizoram	2	2.3
Nagaland	1	1.1
Nepal	2	2.3
Odisha	2	2.3
Sikkim	1	1.1
Tamil Nadu	5	5.7
Tripura	2	2.3
Uttar Pradesh	1	1.1
Uttarakhand	1	1.1
West Bengal	12	13.6

4. Native Kidney Disease characteristics

The frequencies of different diseases have been described in Table 5. A total of 15 different native kidney diseased were identified with unknown aetiology being the commonest.

Table 6: Native Kidney Disease characteristics

NKD	Frequency
1.IgA Nephropathy	16
2.CGN	1
3.Unknown	49
4.Arteriosclerosis	2
5.Diabetic nephropathy	3
6.Bilateral VUR	1
7.Alports	1
8.Chronic Pyelonephritis	1
9.ADPKD	2
10.Obstructive uropathy	5
11.TMA secondary to HTN	1
12.CIN	2
13.Mesangial proliferative disease	1
14.Lupus Nephritis	2
15.ARPKD	1

5. Distribution of graft outcomes in cases:

Cases were classified into various graft outcomes based on biopsy findings into ACR, AMR, ACR+AMR and (TG/ATN/PTC) Transplant glomerulitis with acute tubular necrosis and peritubularcapillaritis.

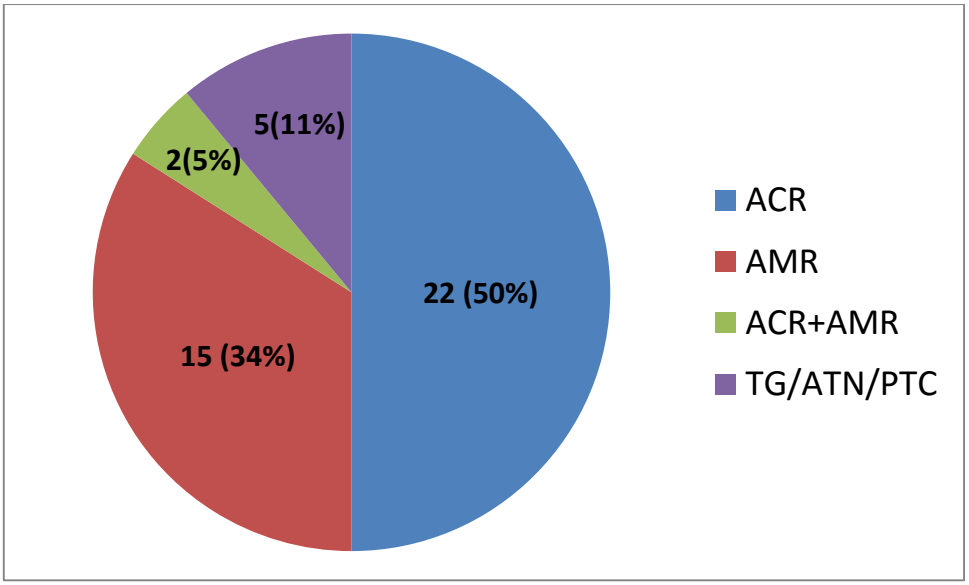


Figure 11: Distribution of graft outcomes in cases

Out of the 44 cases, 22(50%) had ACR, 15(34%) had AMR, 2(5%) had ACR+AMR and 5(11%) had transplant glomerulitis with acute tubular necrosis and peritubular capillaritis.

6. Distribution of timings of rejection episodes in cases tested

Among the cases the time to develop rejection episodes/graft dysfunction from the day of transplantation ranged from 3 days to 180 days with a mean time of 14.8 days duration.

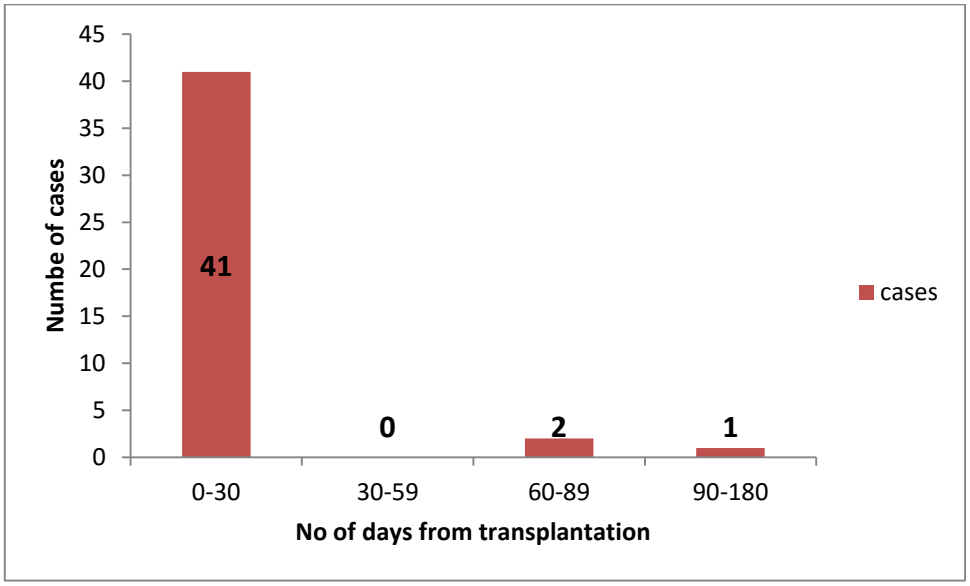


Figure 12: Distribution of timings of rejection episodes in cases tested

7. Prevalence of anti-MICA antibody

7.1 Prevalence in the overall study population

The prevalence of anti-MICA antibody positivity in the overall study population was analysed. Out of the total 88 patients 57 (64.8%) were positive for anti-MICA antibodies, 22 (25.0%) were Negative and 9 (10.2%) were undetermined.

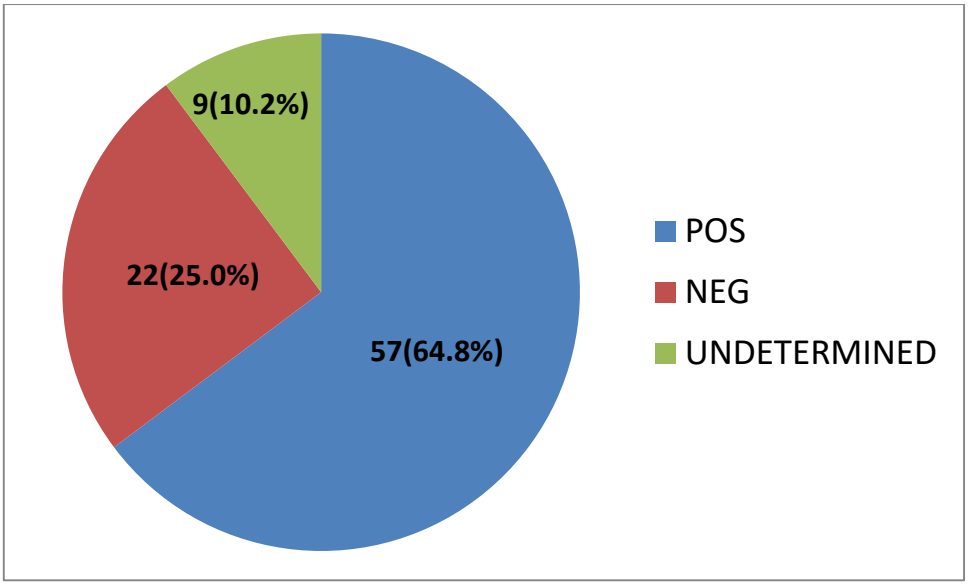


Figure 13: Prevalence of anti-MICA antibody in the overall study population

7.2 Prevalence in the Indian population

Out of a total of 63 patients from India, 41(65.1%) were positive for anti-MICA antibodies, 17 (27.0 %) were Negative and 5 (7.9 %) were undetermined. 14 patients from Bangladesh, 2 patients from Nepal and 9 patients from Bhutan were excluded from calculating the prevalence of anti-MICA antibody in the Indian population.

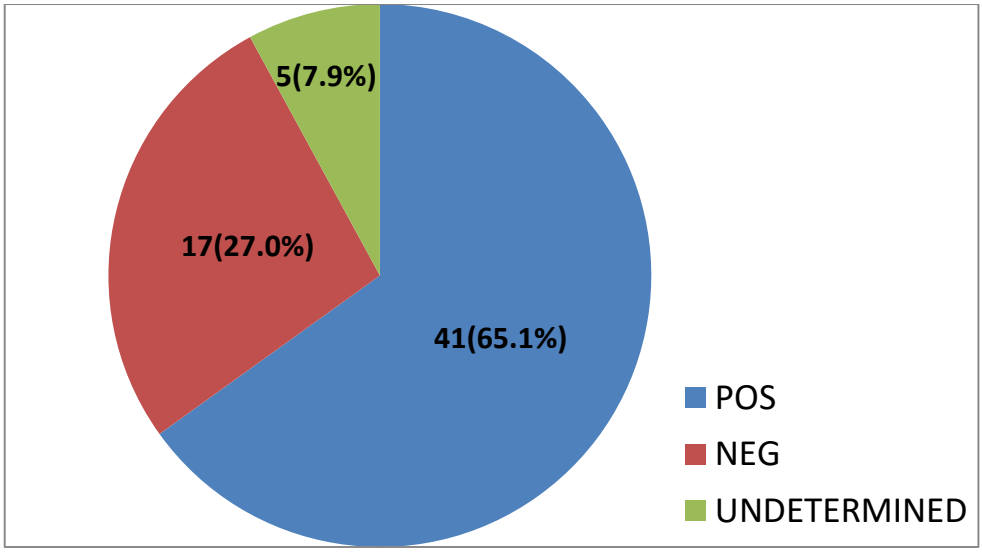


Figure 14: Prevalence of anti-MICA antibody in the Indian population

7.3 Prevalence among cases

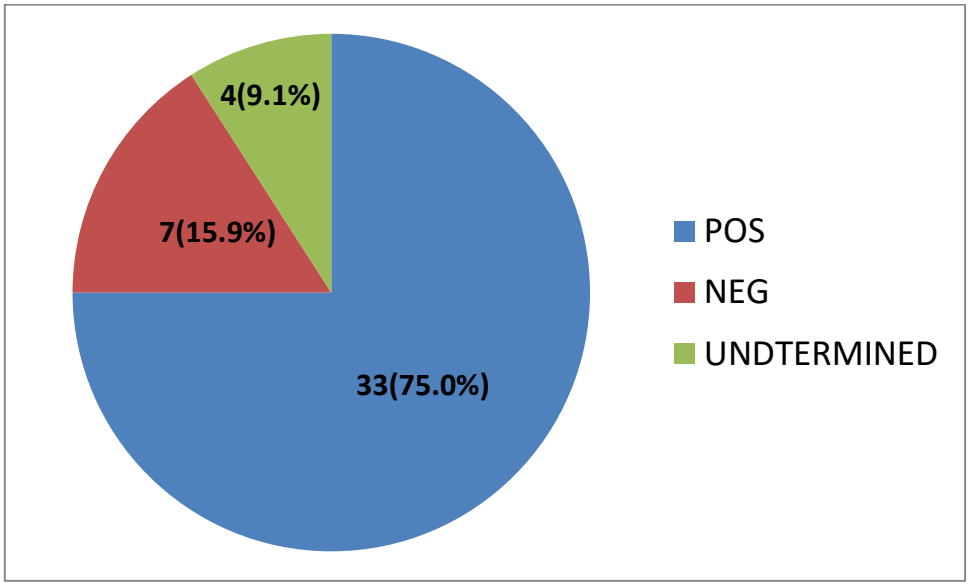


Figure 15: Prevalence of anti-MICA antibody positivity in cases

Out of 44 cases 33(75%) were anti MICA antibody positive,7 (15.9%) were negative and 4 (9.1%) were undetermined.

7.4 Prevalence among Controls

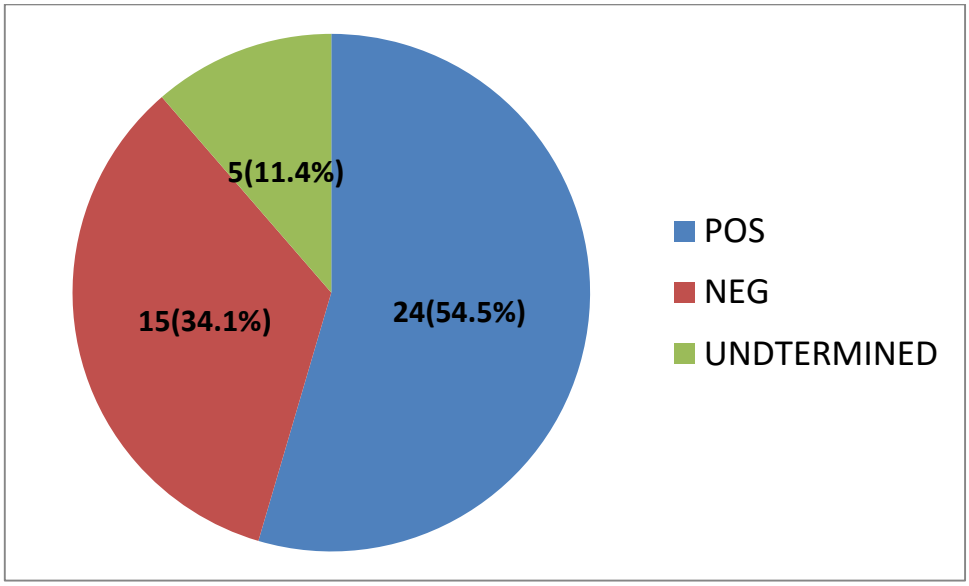


Figure 16: Prevalence of anti-MICA antibody positivity in controls

Out of 44 controls 24 (55%) were anti MICA antibody positive, 15(34%) were negative and 5(11%) were undetermined.

8. Comparison of anti-MICA antibody positivity among cases and controls.

Anti-MICA antibody positivity was compared among cases and controls; the analysis of which has been described in Table 6.

Table 7: Comparison of anti-MICA antibody positivity among cases and controls.

	Anti-MICA Ab POS N (%)	Anti-MICA Ab NEG N (%)	p value
case	33(75)	7(15.9)	0.038
control	24(54.5)	15(34.1)	

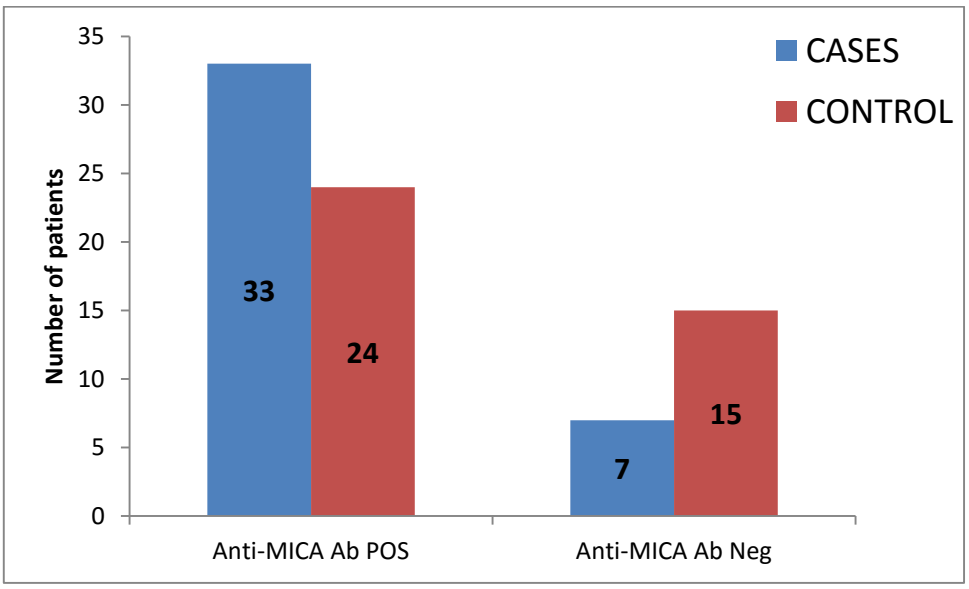


Figure 17: Comparison of anti-MICA antibody positivity between cases and controls

On comparison between cases (75%) and controls (54.5%) **anti MICA antibody positivity is significantly higher in cases with a p value of 0.038** which was calculated using Pearson's chi square test. 4 patients among cases and 5 among controls were undetermined in terms of anti-MICA antibody positivity , which were excluded from the above p value calculation.

9. Anti MICA antibody positivity in various graft outcomes

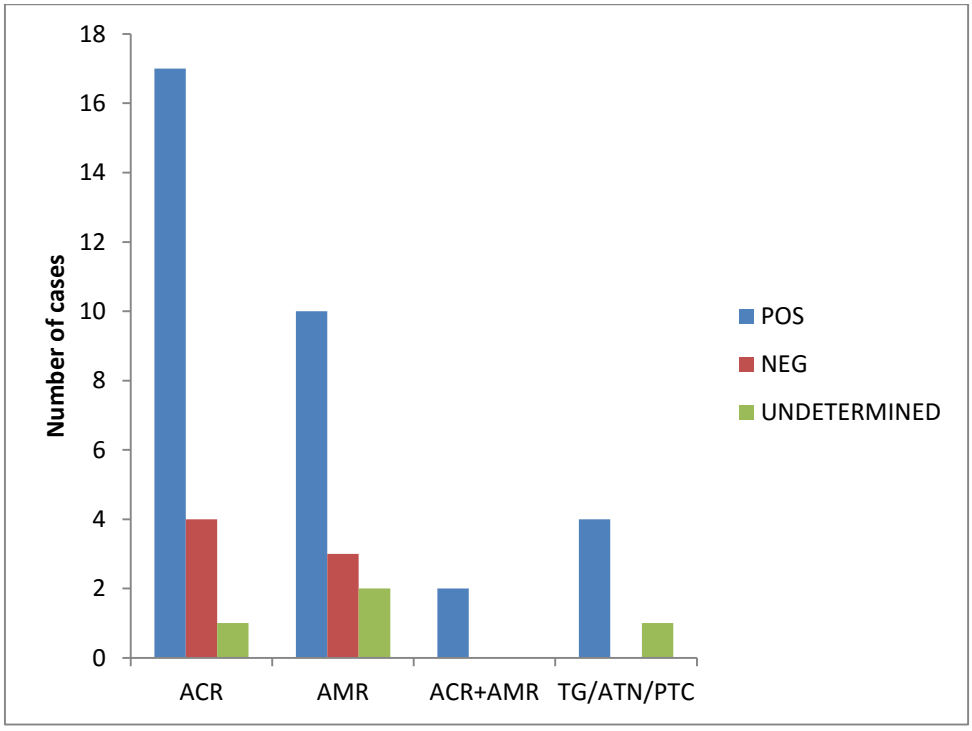


Figure 18: Anti MICA antibody positivity in various graft outcomes

Correlation of Anti MICA antibody positivity with varied graft outcomes was analysed in the 44 cases tested. A large proportion of cases with ACR (17/22 ;77%) , AMR (10/15 ;67%) , all cases with combined ACR+ AMR (2/2 ;100%) and a large proportion of transplant glomerulitis +ATN+ peritubular capillaritis (4/5; 80%) were anti MICA antibody positive.. Of the 5 cases of transplant glomerulitis with peritubular capillaritis three had acute graft dysfunction and the other two had slow graft recovery.

10. NBG RATIOS IN CASES AND CONTROLS

Positivity of anti-MICA antibody was expressed as Normalised Background Ratio (NBG ratio).The cut offs of NBG ratio provided by One Lambda Inc is interpreted as follows.

<1.2: negative

1.2- 1.5: undetermined

>1.5: positive

In our study NBG ratios varied from 0.10 to 75.45. In an attempt to see if there was any association of graft outcome / types of graft failure with differing levels of NBG ratios, we grouped patients into those with NBG ratios <1.2, 1.2-1.49, 1.5 – 5 and those with NBG ratios >5.

NBG ratio was compared among cases and controls. In the groups NBG<1.2 and NBG between 1.2 and 1.49, larger numbers of controls were seen, as opposed to the groups with NBG 1.5-5 and >5, where cases predominated.

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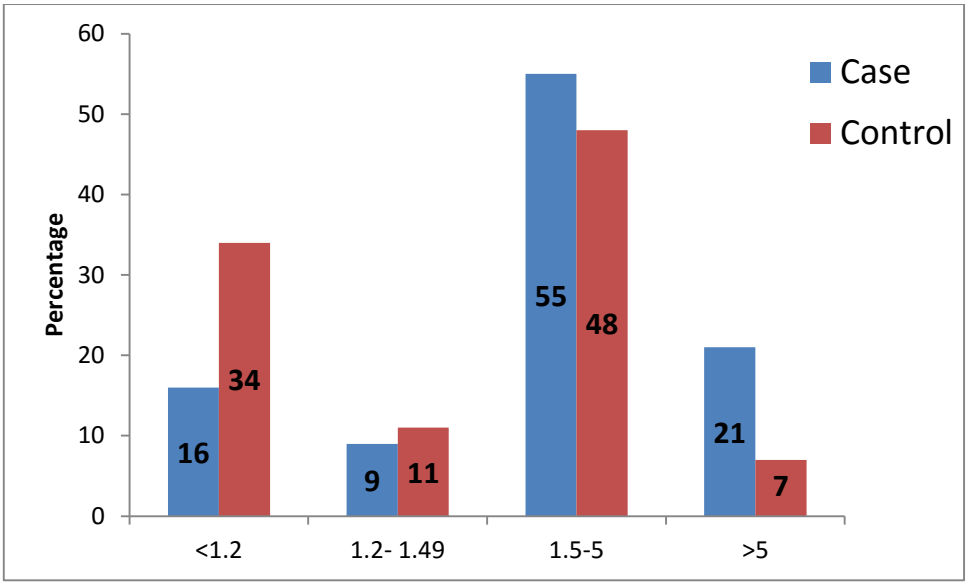

Figure 19: NBG Ratios among cases and controls

Graft outcome was compared amongst the different NBG ratio groups mentioned above. The Odd's ratio with 95% CI for poor graft outcome is presented

Is presented in table 7.

Table 8: Risk for poor graft outcome among different NBG ratio groups

	case	Control			
NBG	N(%)	N(%)	OR	95%CI	P VALUE
<1.2	7(16)	15(34)	REF		
1.2-1.49	4(9)	5(11)	1.71	0.35,8.42	0.507
1.5-5	24(55)	21(48)	2.45	0.84,7.15	0.101
>5	9(21)	3(7)	6.43	1.32,31.37	0.021

Patients with NBG ratio >5 had 6.43 times higher risk for developing poor graft outcome in comparison to those patients who had NBG ratio <1.2. This was statistically significant (p=0.021).

11. Various biopsy proven graft outcomes and NBG ratios

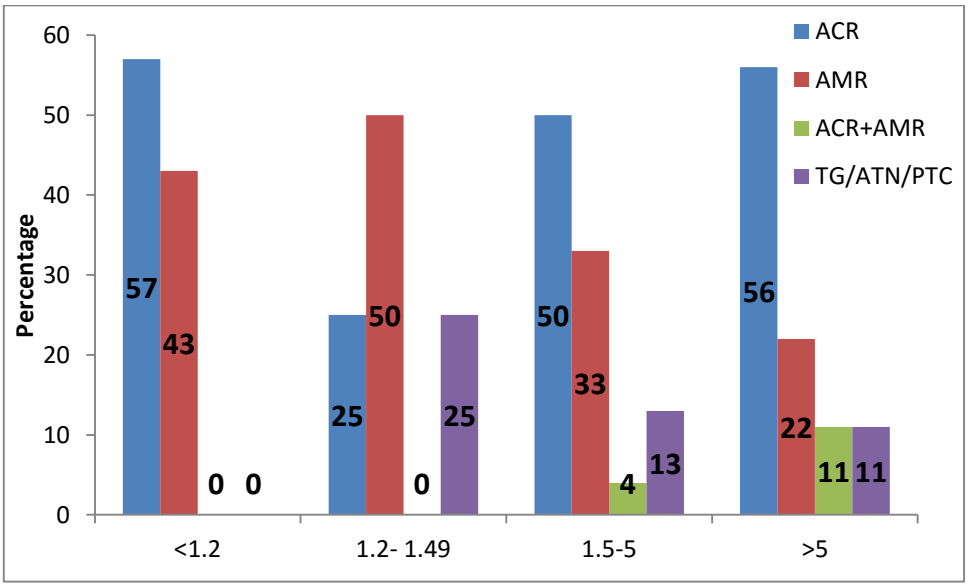

Figure 20: Various biopsy proven graft outcomes and NBG ratios

Various graft outcomes were analysed across 4 different groups of NBG ratios. It was observed that ACR predominated in the NBG ratio groups 1.5-5 (50%) and >5 (56%) in comparison to other graft outcomes.

12. Anti-MICA antibody positivity in ACR

The Risk for developing ACR in patients among different NBG ratio groups was analysed which are described in Table 8.

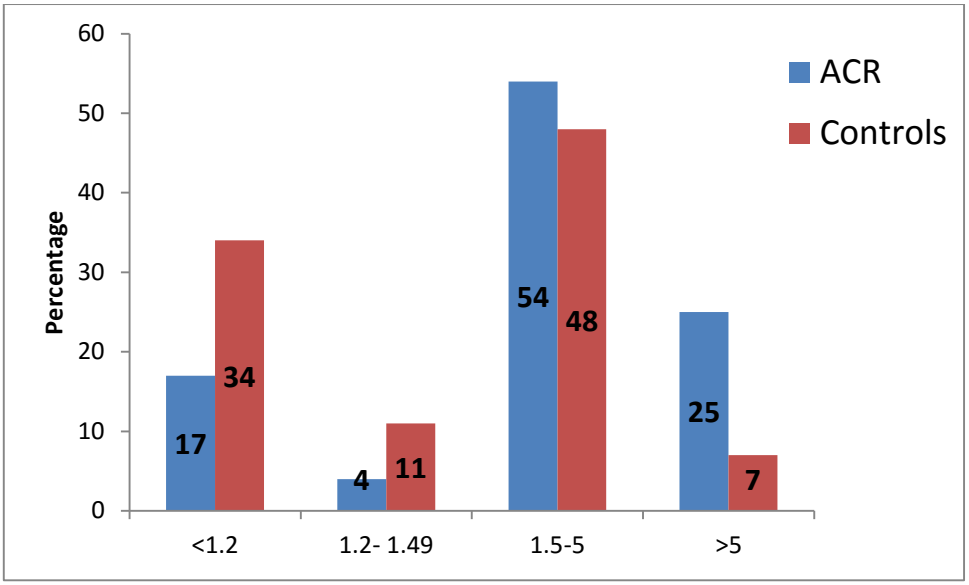

Figure 21: Anti-MICA antibody positivity in ACR

Table 9: Risk for ACR among different NBG ratio groups

NBG	ACR (%)	CONTROLS (%)	OR	95% CI	P VALUE
<1.2	4(17%)	15(34%)	REF		
1.2-1.49	1(4%)	5(11%)	0.75	0.07,8.38	0.815
1.5-5	13(54%)	21(48%)	2.32	0.63,8.53	0.205
>5	6(25%)	3(7%)	7.50	1.27,44.08	0.026

Calculated odds ratios showed that patients with NBG ratio 1.5- 5 group had 2.32 times higher risk for ACR. Patients with NBG ratio >5 had 7.50 times higher risk for developing ACR compared to the group having NBG ratio <1.2 which was also statistically significant. (p=0.026).

13. Anti-MICA antibody positivity in AMR

The Risk for developing AMR in patients among different NBG ratio groups was analysed which are described in Table 9.

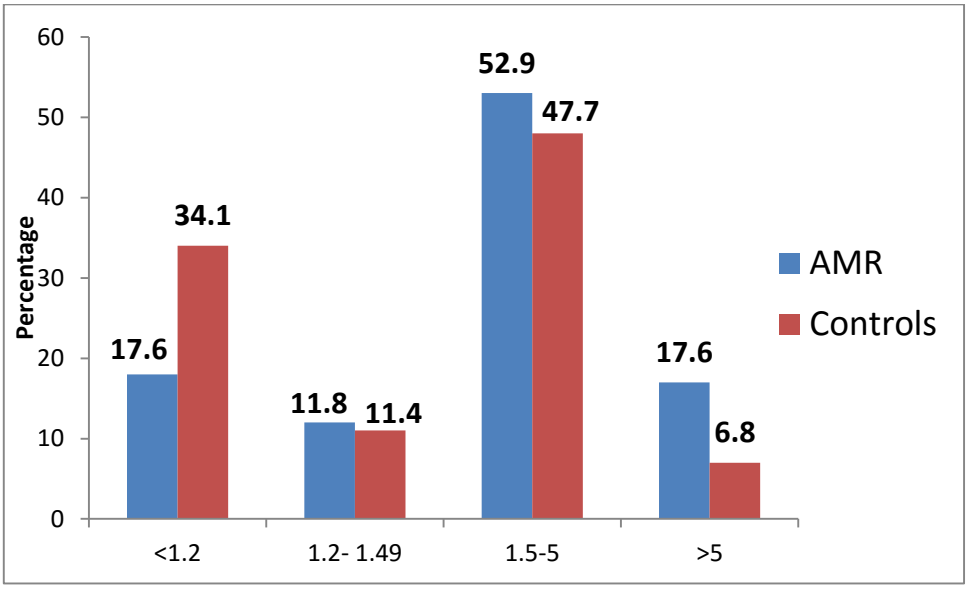

Figure 22: Anti-MICA antibody positivity in AMR

Table 10: Risk for AMR among different NBG ratio groups

	AMR(%)	controls	OR	95%CI	P VALUE
<1.2	3(17.6%)	15(34.1%)	REF		
1.2-1.49	2 (11.8 %)	5(11.4%)	2.00	0.26.15.62	0.509
1.5-5	9(52.9 %)	21(47.7%)	2.14	0.49,9.27	0.308
>5	3(17.6 %)	3(6.8 %)	5.00	0.66,37.85	0.119

Calculated odds ratio showed the group with NBG ratio 1.5- 5 had 2.14 times higher risk for AMR and the group with NBG ratio>5 had 5 times higher risk for AMR compared to NBG ratio <1.2 .

14. Correlation of anti-mica antibody with sensitising events.

a) Transfusion and anti-MICA antibody

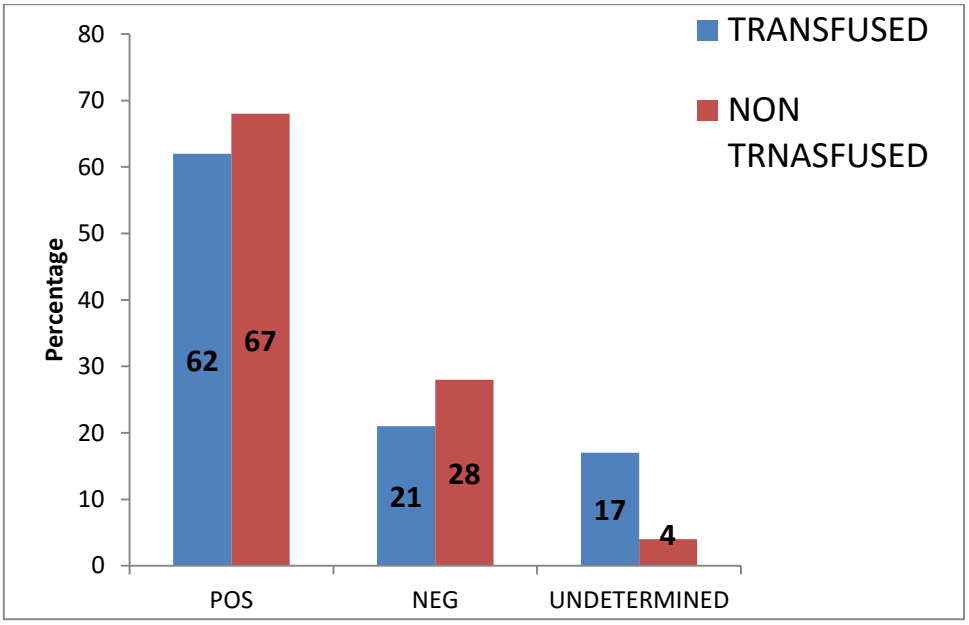

Figure 23: Transfusion and anti-MICA antibody

Out of all 88 patients 42 patients had history of prior transfusion. Among the patients with transfusion 61.9 %(n= 26) showed anti- MICA antibody positivity and 21.4% (n=9) were negative. 16.67% (n=7) patients had undetermined NBG ratio. Among the non-transfused patients 67.4% (n=31) showed anti- MICA antibody positivity, 28.3 %(n=13) showed negativity and 4.3 %(n=2)showed undetermined NBG ratio. There was no statistically significant association found between prior transfusion and anti-MICA antibody positivity (p=0.706).

b) Pregnancy and Anti-MICA antibody

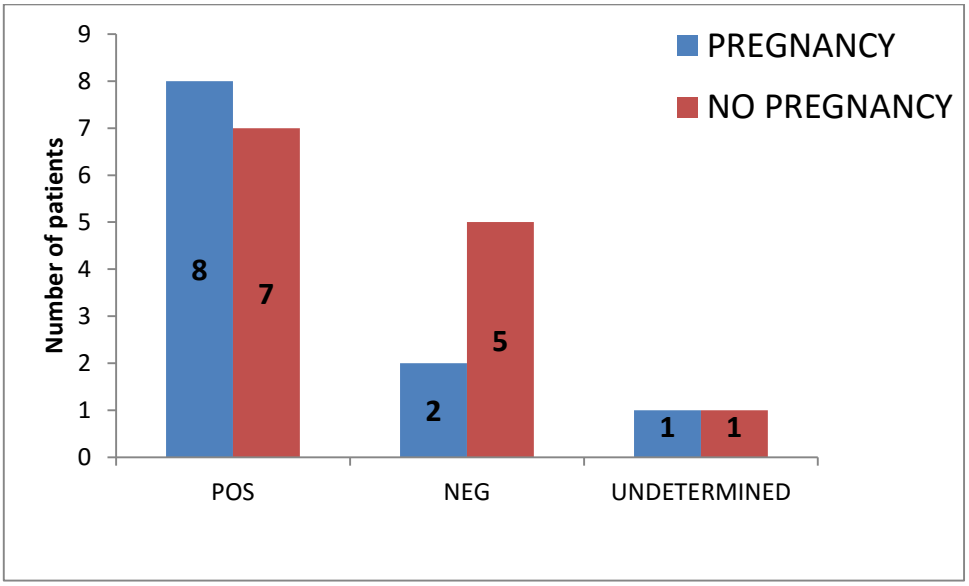

Figure 24: Pregnancy and anti-MICA antibody

Correlation between pregnancy and anti-MICA antibody positivity was analysed. Out of 24 female patients 11 had history of pregnancy. Among patients with history of pregnancy 72.7 %(n=8) patients showed anti-MICA antibody positivity, 18.18% (n=2) showed negativity and 9 %(n=1) showed undetermined NBG ratio. Among 13 female patients with no history of pregnancy 53.84 %(n=7) showed anti-MICA antibody positivity, 38.46 %(n=5) showed negativity and 1(7.69%) showed undermined NBG ratio. Hence there was no significant association found between pregnancy and anti-MICA antibody positivity (p = 0.381).

DISCUSSION

As HLA is a major target of preformed antibodies in the transplant recipient causing allograft dysfunction and rejection the current practice is to ensure there are no donor specific anti HLA antibodies (DSA) in the transplant recipient. However, even in the absence of HLA DSA, cases of antibody mediated allograft dysfunction, poor graft function and rejections are increasingly reported. Among the potential non HLA antigens known to impact on renal allograft dysfunction MICA, a highly polymorphic non HLA histocompatibility antigen located extremely close to the HLA B locus, appears to impact. Despite increasing literature highlighting the possible role of anti-MICA antibodies emerging in the context of renal transplantation and renal allograft injury, there are very few studies in the Indian patient population awaiting renal transplant. Against this background our study was performed.

The prevalence of Anti MICA antibodies in our overall study population of 88 patients, and the Indian patient group of 63 patients was comparable (64.8 and 65.1% respectively). The prevalence in our study is considerably higher than that reported in many other studies from both India and overseas. A study from India by Chowdhry et al (112) reported a prevalence of 14.6% for anti-MICA antibodies in a cohort of 646 patients tested. The LSA MICA kit was used in this study for detection of anti HLA antibodies. In a study by Sumitran et al (7) 139 pre-renal transplantation serum samples were assayed for anti-MICA antibodies and 37 out of 139 (26.6%) samples showed anti-MICA antibody positivity. Another study by Lemy et al (92)where the prevalence of anti-MICA Antibody was assayed using Luminex single antigen assay

(LSA-MICA) among a cohort of 1091 individuals which included 494 healthy individuals and 597 CKD stage V patients, the prevalence of anti-MICA antibody was 14.9% in CKD patients and 4.7% in healthy individuals. In the same study one third of patients with CKD showed positivity for anti-MICA antibodies. They have also observed that 20% of CKD patients were detected to have anti-MICA antibodies that were auto-reactive (92). The latter finding could not be commented on in our study as MICA typing and specificity of the anti-MICA antibody detected was not tested for.

A study by Sanchez - Zapardiel et al (108) where 727 patients awaiting renal transplant were tested, showed the prevalence of anti-MICA antibody to be 7.5%. Zou et al has described the prevalence of anti-MICA antibody in post-transplant sera of transplanted patients as 22.7%(15 out of 66), in pre transplant sera of patients awaiting transplant as 24.7% (21 out 85) and identified it in 18.6% (11 of 59) of acid eluates from nephrectomy specimens – all tested on the Luminex platform (117).

Analysing the difference in prevalence of anti-MICA antibodies in cases, and controls, our study showed a significant association of anti-MICA antibodies with poor graft outcome , evidenced by 75% of patients vs 54% of controls showing the presence of anti-MICA antibodies. This difference was statistically significant ($p<0.038$). Our results are very similar to many studies in literature. Mizutani and Terasaki et al studied 63 cases of renal allograft rejection and 82 controls with good graft outcome. They showed that 52 % cases and 21 % of controls showed anti- MICA antibody positivity , a difference that was statistically significant ($p<0.001$).(113).

In the study by Sumitran et al 81.1% of patients with anti-MICA antibodies in pre transplant serum developed acute graft rejection in contrast to 38.2% negative for the same ($p<0.001$)(7).

Analysing data from the study performed by Zou et al, (91) pre transplant anti-MICA antibody positivity was associated with increased allograft loss. The mean 1 year graft survival rate among recipients with pre transplant anti-MICA antibody positivity was 88.3±2.2% as opposed to 93.0±0.6% among those recipients with no anti-MICA antibody positivity ($P=0.01$). This difference in percentage of survival among antibody positive and negative groups was maintained at 5 years after transplantation. The survival rate was even lower among anti-MICA antibody positive first time renal transplant patients (87.8±2.4%) than among anti-MICA antibody–negative patients. (93.5±0.6%, $P = 0.005$)(91). In addition to this, the poor graft outcome associated with anti-MICA antibody positivity was more evident in those transplant recipients with good HLA matching (0 or 1 mismatch in HLA-A and HLA-B and HLA-DR). The Hazard ratio for developing graft rejection in this group with anti-MICA antibody positivity was 5.19 with $p=0.001$ in comparison to those who were negative for anti-MICA antibody. In the above mentioned group the graft survival was 83.2+/-5.8% among those who were positive for anti-MICA antibodies as opposed to 95.1+/-1.3% among those who were negative for anti-MICA antibodies, $P=0.002$

Although there were frequent acute rejection episodes with features of AMR among patients with anti-MICA antibody positivity (5 out of 6 as opposed to 19 out of

47,p=0.08) Lemy et al observed a better overall 10 year survival in patients with pre transplant anti MICA antibody positivity than patients who were tested negative for anti-MICA antibodies. 10 year overall graft survival was 88% for patients who were positive for anti-MICA antibody as opposed to 74% in patients who were negative which was statistically not significant (88% vs. 74%, P= 0.25) (92). The authors comment that this observation can be probably attributed to the fact that the patients in that study were under higher immunosuppression than patients included in Zou et al's study.

Our study is also in keeping with the findings of Chowdhry et al where in a cohort of 646 renal pre transplant recipients with isolated anti-MICA antibody positivity in pre transplant serum and no anti-HLA antibodies, they observed greater numbers of patients with poor graft outcome.((hazard ratio of 2.768, P<0.05) . Patients who tested positive for anti-MICA antibody had 89.3% graft survival as compared to 94.7% in patients who tested negative for anti-MICA antibody (P<0.05). (112).

In our study the mean time to develop rejection/graft dysfunction among cases was 14.8 days with a range of 3 to 180 days suggestive of predominance of acute graft rejections. This observation is keeping in line with that of study by Sumitran et al where 81.1.% of patients with pre transplant anti-MICA antibody positivity developed acute graft rejection in contrast to 38.2% negative for the same(p<0.001) (7). Similarly Cox et al (95) and Narayan et al (109) has also observed the association of acute graft rejection and anti-MICA antibody positivity as discussed below.

Although the earlier studies on MICA had described its role in AMR (7,106), in our study a very strong correlation was observed with pre transplant anti-MICA antibody positivity and the risk of developing ACR. It appears that anti-MICA antibodies can be implicated in both cellular and humoral immunity. A Similar observation was also made earlier by Cox et al who observed that in a cohort of 391 renal transplant recipients biopsied, 26 were anti-MICA antibody positive, while 365 tested negative. 35 % of patients positive for anti-MICA antibodies developed ACR as opposed to 17% who were negative (p=0.021). They also documented that the presence of both anti HLA and anti-MICA antibody showed significant association with ACR with a calculated Odd's ratio of 4.9 and p value of 0.018(95) The suggested mechanism for this observation was that T-cell indirect allorecognition of mismatched MICA epitopes gives rise to the cell-mediated immunity resulting in cellular rejection and that it could also co stimulate helper T-cell which in turn stimulate B cells and play a role in the production of IgG antibodies against MICA(95).

Similarly, in a case report by Narayan et al donor specific anti MICA antibody positivity was associated with AMR and Banff type IIA ACR. A high titre pretransplant anti MICA antibody MFI of >15000 was observed in the described case underscoring the importance of quantification and monitoring of anti-MICA antibody levels in those patients with anti-MICA antibody positivity(109).

Unlike HLA antigens, MICA antigens are not expressed on peripheral lymphocytes but on the surface of endothelial cells, keratinocytes, monocytes and dendritic cells .Transfusion of blood products, pregnancy and organ transplantation are the described factors implicated in anti-MICA antibody production similar to anti HLA antibodies

(118). However, in our study no significant association was observed between transfusion (p = 0.706) or pregnancy (p =0.381) with anti-MICA antibody production This is similar to the observation made by Zapardiel et al (108). This observation implies that pre transplantation sensitization against MICA and HLA occur independently as evidenced by anti-MICA antibody positivity in patients with or without HLA antibodies. Being a protein which has enhanced expression in biological stress conditions, viral infections, tumour transformation and autoimmune diseases can potentially incite anti-MICA antibody production. Further studies will be required to draw more definitive associations and conclusions.

Lemy et al has described male gender as an independent risk factor for developing anti MICA antibodies (92). However in our study 73.7% of males and 68.2 % of females were anti-MICA positive showing no gender correlation and anti-MICA antibody positivity (p =0.625). However, given the limited sample size, and the predominant male population in our study, it is hard to draw conclusions on our data. It would require gender matched studies with a larger sample size

Anti-MICA antibody positivity in our study is expressed as NBG ratio. There is no literature available on the significance of NBG ratios of anti-MICA antibody positivity and its impact on various graft outcomes. Traditionally, it has been expressed as MFI, and a case report by Narayan et al (109) has shown that anti-MICA with MFI >15000 to be associated with acute rejection. Similarly , Mehra et al also described that preformed donor specific anti MICA antibodies of higher MFI (10,000-20,000) can cause hyper acute to acute renal allograft rejection(110). Ming et al

describes donor specific anti-MICA antibodies with MFI >10,000 as a risk factor for acute antibody mediated rejection (111) especially for re transplants.

There is emerging literature on NBG ratio as being indicative of the strength of anti HLA antibodies in studies of patients with GVHD (115) and TRALI (119). On the other hand, Fadei et al have attempted to use different cut offs in NBG ratios identified, on HLA screening tests and correlate them with the SAB assay. They showed that cut offs of 10.8 for class I and 6.9 for class II correlated best with the SAB as opposed to the cut off in the product insert which was 2.2. Using the latter would have had implications of false positivity on the screening assay. (116) This study was performed on platelet apheresis donors.

Our study showed a wide range of NBG ratios on the patients tested. We are aware that LABScreen Mixed antigen test is a qualitative assay. However in view of the emerging literature on high NBG ratios impacting clinically, we stratified NBG ratios into 4 groups. - NBG<1.2, NBG ratio 1.2 -1.49, NBG ratio 1.5-5 and NBG ratio >5, It is interesting to note that amongst those reported as positive for anti-MICA antibodies with NBG ratios >1.5 as the cut off, a very differential pattern was seen between cases and controls. In the groups with NBG 1.5-5 and >5, cases clearly predominated. We showed also that patients with NBG ratio >5 carry 6.43 times higher risk for developing poor graft function (OR of 6.43, p=0.021)

Analysing NBG ratio associations in the context of ACR and AMR, we observed that patients with NBG ratio >5 had 7.5 times higher risk of developing ACR in comparison to anti-MICA antibody negativity (NBG ratio <1.2) (OR=7.5.p=0.026). and calculated Odd's ratio in patients with NBG ratio of 1.5-5 showed 2.32 times

increased risk of developing ACR.(OR=2.32,p=0.205). In AMR, the trend though similar, was not as pronounced, nor did it reach statistical significance even in the group of cases with NBG ratio >5.

Ensuring longevity of renal allografts is critical to any transplant program. Particularly in resource constrained settings where re transplant can pose significant financial and logistic challenges, it is critical to ensure adequate pre transplant testing to minimise graft injury. Our study, though limited by numbers, shows a prevalence of Anti MICA antibodies significantly higher than that reported in literature. Given the association with poor graft outcome in our study, which is consistent with many studies in literature, our data suggests that incorporating this into the pretransplant algorithm of tests, might enable identifying a subset of patients, negative for anti HLA antibodies, who may be at significant risk for post-transplant graft injury. Identification of this risk factor will facilitate prophylactic/early intervention / close follow up / and appropriate consideration during tailoring of immunosuppression – all factors being critical to enhance graft outcome and enhanced quality of patient care.

Limitations:

- Anti-MICA antibody positivity was tested using a screening assay and donor specificity of the anti-MICA antibody was not ascertained.
- As the study design was an unmatched case control study, cases and controls were not comparable for all variables and the possibility of potential confounders cannot be excluded.
- Sample size of our study was small in comparison to many other studies in the literature.
- Though the luminex platform was used the XmDSA with its inherent limitations and the CDC crossmatch were used to determine HLA antibody status.
- The possibility of coexisting non donor specific HLA antibodies in these patients cannot be excluded

CONCLUSION:

- The prevalence of anti-MICA antibody is significantly higher in our patient population in comparison to that described in literature.
- Pre transplant anti-MICA antibody positivity is significantly associated with poor graft outcome.
- The mean time to rejection / graft dysfunction was 14.8 days.
- Pre transplant Anti MICA antibody positivity is significantly associated with ACR
- NBG ratio >5 is significantly associated with ACR.
- There was no association of anti-MICA antibody positivity with prior transfusions or pregnancy.

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March 29, 2017

Dr. Blessymol Varghese,
PG Registrar,
Department of Transfusion Medicine,
Christian Medical College,
Vellore - 632 004.

Sub: **Fluid Research Grant NEW PROPOSAL:**
Pre transplant MICA antibodies and impact on renal allograft outcome.
Dr. Blessymol Varghese, Employment Number: 21299, PG Registrar, Dr. Dolly Daniel Employment number: 11674, Professor, Dr. Mary Purna Chacko, Employment number: 31611, Professor, Dr. Pragya Kafley, Employement Number: 21143, PG registrar, Mr. Sam Arul Doss , Employment number: 32377, Research demonstrator, HLA laboratory, Department of Transfusion Medicine and Immunohematology, Dr. Santosh Varghese, Employment number: 28219, Professor& Head, Dr. Vinoi George David, Employment number: 31475, Professor, Dr. Jagdish. K, Employment number: 21157, Senior PG registrar, Ms. Tunny Sebastian, Employment Number: 32291, Lecturer, Department of biostatistics.

Ref: IRB Min No: 10464 [OBSERVE] dated 05.01.2017

Dear Dr. Blessymol Varghese,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Dolly Daniel, Dept. of Transfusion Medicine, CMC, Vellore

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

March 29, 2017

Dr. Blessymol Varghese,
PG Registrar,
Department of Transfusion Medicine,
Christian Medical College,
Vellore - 632 004.

Sub: **Fluid Research Grant NEW PROPOSAL:**
Pre transplant MICA antibodies and impact on renal allograft outcome.
Dr. Blessymol Varghese, Employment Number: 21299, PG Registrar, Dr. Dolly Daniel Employment number: 11674, Professor, Dr. Mary Purna Chacko, Employment number: 31611, Professor, Dr. Pragya Kafley, Employement Number: 21143, PG registrar, Mr. Sam Arul Doss , Employment number: 32377, Research demonstrator, HLA laboratory, Department of Transfusion Medicine and Immunohematology, Dr. Santosh Varghese, Employment number: 28219, Professor& Head, Dr. Vinoi George David, Employment number: 31475, Professor, Dr. Jagdish. K, Employment number: 21157, Senior PG registrar, Ms. Tunny Sebastian, Employment Number: 32291, Lecturer, Department of biostatistics.

Ref: IRB Min No: 10464 [OBSERVE] dated 05.01.2017

Dear Dr. Blessymol Varghese,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Pre transplant MICA antibodies and impact on renal allograft outcome" on January 05th 2017.

The Committee reviewed the following documents:

1. IRB Application format
2. Patient Information Sheet
3. Cvs of Drs. Blessy, Jagdish, Dolly, Purna, Santhosh, Vinoi, Pragya, Tunny Sebastian and Mr. Sam.
4. No. of documents 1 – 3

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on January 05th 2017 in the BRTC Conference Room, Christian Medical College, Bagayam, Vellore 632002.

2 of 4

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Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA (Theology), Dr. Min (Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Rekha Pai	BSc, MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal,Basic Medical Scientist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist &Epidemiologist
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician

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Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Santhanam Sridhar	MBBS, DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MBBS, MD, DNB, PhD	Professor,Endocrinology, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr Sneha Varkki	MBBS, DCH, DNB	Professor, Paediatrics, CMC, Vellore	Internal, Clinician
Dr. Sathish Kumar	MBBS, MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Pre transplant MICA antibodies and impact on renal allograft outcome"on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:

A sum of 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 10464 [OBSERVE] dated 05.01.2017

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Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

DATA SPREAD SHEETS (CASES)

SERUMNHosp No	AGE	GENDER_1M_2F	Tx date	Type_1L_HLA	MATTRANSFUNO. OF TY	PREGNAI	DONOR	RELATIONINID	INCO_1gc	Biopsy	Biopsy_r	ClinicalD	cDiDiag_r	CDC	DSA	CLASSDSA	CLASSDSA	CLASSDSA	CLASSA,B	FLOW	CLASS I	CLASS1	CLASS1	CLASS I_	MICA	MICA	PC/NC	N86 Rati	group
4590 2955930G	33	1 BANGLAD	09-03-2016	1 4|8		1	0 33|M	BROTHER	CGN	2 AMR	1			NEG	1 NEG	1 NEG	1		1	NEG	1 NEG		1 NEG		1	575.42	0.8291	1
4691 9740S4F	20	1 BANGLAD	19-04-2016	1 5|8		1	0 46|M	FATHER	AJPORT'S	7 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 NEG		1 POS		2	115.6	1.891	1
3016 0089567F	26	1 TAMILNAI	10-12-2016	2 3|8	4	1	0 57|M	UNRELATE	IgA;NEPHF	1 AMR	1			NEG	1 NEG	1 NEG	1			NEG	1 NEG		1 UNDETER		3	347.13	1.3292	1
3541 7636D2F	30	1 BIHAR	27-11-2014	1 0|8	6	1	0 29|F	WIFE	UNKNOWI	3 AMR	1			NEG	NEG	1 NEG	1			POS	2 UNDETER		3 POS		2	106.11	2.3996	1
0 0680704F	44	1 BANGLAD	30-07-2014	1 1|8		1	0 36|F	WIFE	IgA;NEPHF	1 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	646.8	3.5114	1
3740 1350B4F	49	1 KERALA	01-10-2014	2 2|8		1	0 31|M	UNRELATE	IgA;NEPHF	1 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	537.04	3.9194	1
3780 8594B1D	39	1 ANDHRA F	24-10-2014	2 NA	5	1	0 40|M	UNRELATE	ARTERIOS	4 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	870.94	2.7783	1
3970 2595A7D	33	1 ANDHRA F	07-02-2015	2 NA	6	2	0 31|M	UNRELATE	IgA;NEPHF	1 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		1	199.01	0.7456	1
4454 0793106G	59	1 JHARKHAI	19-11-2015	1 2|8	4	1	0 56|F	WIFE	DIABETIC	5 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	406.1	2.0368	1
4455 9737135F	31	1 JHARKHAI	22-12-2015	1 0|8	3	1	0 26|F	WIFE	UNKNOWI	3 AMR	1			NEG	1 NEG	1 NEG	1			NEG	1 NEG		1 POS		2	386.71	27.8622	1
4535 2067710G	22	1 SIKKIM	27-01-2016	1 4|8	2	1	0 54|M	FATHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			NEG	1 NEG		1 POS		2	497.2	66.0072	1
4591 2119D6G	28	1 BANGLAD	25-02-2016	1 5|8		1	0 53|F	MOTHER	UNKNOWI	3 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	479.03	2.4244	1
3899 0116L0G	29	1 BANGLAD	15-01-2015	1 7|8	1	1	0 46|F	MOTHER	UNKNOWI	3 TG/ATN/P	3 SLOW GR/	3 NEG			1 NEG	1 NEG	1	NEG	NEG	POS	1 POS		2 POS		2	1072.54	1.792	1
4644 0479306G	26	1 BANGLAD	24-03-2016	1 1|8	1	1	0 36|M	COUSIN	UNKNOWI	3 TG/ATN/P	3 A/C Graft dysfunction	NEG			1 NEG	1 NEG	1			POS	2 NEG		1 UNDETER		3	123.75	1.2384	1
4673 9826B7F	31	1 BANGLAD	13-04-2016	1 4|8	3	1	0 49|F	MOTHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 NEG		1 POS		2	486.64	3.0948	1
4706 3763B1G	35	1 NEPAL	23-06-2016	1 1|8		1	0 33|F	WIFE	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	378.13	3.5732	1
4793 3442F3G	45	2 CHATTISG	07-06-2016	1 1|8		1	2 47|M	HUSBAND	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 NEG		1 NEG		1	327.76	0.9092	1
4866 0307B4G	22	1 CHATTISG	17-08-2016	1 4|8	3	1	0 45|F	PATERNAL	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 POS		2 NEG		1	121.98	0.9272	1
4870 1697J1A	39	1 JHARKHAI	31-08-2016	2 1|8	1	1	0 45|M	UNRELATE	B/L VOR	6 AMR	1			NEG	1 NEG	1 NEG	1 NEG			POS	2 POS		2 UNDETER		3	277.85	1.1286	1
4952 4077415	36	1 ORISSA	22-09-2016	1 0|8	2	1	0 39|F	SISTER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 BP	3	NEG	POS	POS	2 POS		2 POS		2	143.92	4.3942	1
4962 0657B8C	52	1 MEGHALA	11-10-2016	1 1|8		1	0 47|F	COUSIN	IgA;NEPHF	1 ACR	2			NEG	1 NEG	1 NEG	1			NEG	1 NEG		1 POS		2	2377.7	2.3018	1
3981 8580E9F	50	1 BHUTAN	19-02-2015	1 1|8		1	0 38|F	WIFE	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			UNDETER	3 NEG		1 NEG		1	388.05	0.9253	1
4228 9368A0F	42	1 WEST BEN	17-06-2015	1 3|8		1	0 39|F	WIFE	UNKNOWI	3 ACR	2			NEG	1 NEG	NEG	1			POS	2 POS		2 POS		2	115.73	2.7008	1
4314 9553A3F	26	1 TAMILNAI	12-08-2015	1 5|8		1	0 55|M	FATHER	UNKNOWI	3 AMR	1			NEG	1 NEG	1 BP (559)	3			POS	2 POS		2 POS		2	346.39	1.9878	1
4947 2128D7G	27	1 BANGLAD	02-09-2015	1 4|8	6	1	0 52|M	FATHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 BP (511)	1			POS	2 UNDETER		3 UNDETER		3	346.46	1.272	1
4376 1694B4G	41	1 UTTAR PR	23-09-2015	1 1|8		1	0 38|F	WIFE	UNKNOWI	3 AMR	1			NEG	1 NEG	1 BP	3			POS	2 POS		2 NEG		1	152.69	0.8932	1
4419 2442A5G	40	1 TAMILNAI	27-10-2015	1 4|8		1	0 62|M	FATHER	UNKNOWI	3 AMR	1			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	144.18	5.5456	1
4446 9201193F	28	1 CHATTISG	11-11-2015	1 4|8	8	1	0 40|F	MOTHER	C/C PYELO	8 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 POS		2 POS		2	107.35	77.195	1
4526 2948D2g	40	1 BHUTAN	01-06-2016	1 8|8		1	0 25|F	SISTER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 BP	3			POS	2 POS		2 POS		2	230.69	2.607	1
4534 3337J1G	18	1 CHATTISG	13-01-2016	1 6|8		1	0 48|F	MOTHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1 NEG			NEG	1 UNDETER		3 POS		2	376.72	4.015	1
4579 9670L1F	30	1 WEST BEN	17-02-2016	1 4|8	1	1	0 52|F	MOTHER	IgA;NEPHF	1 AMR+ACR 2+1				NEG	1 NEG	1 BP	3			POS	2 POS		2 POS		2	547.83	51.6948	1
4601 1736H7G	48	1 BANGLAD	01-03-2016	1 5|8		1	0 62|F	MOTHER	ADPKD	9 TG/ATN/P	3 A/C Graft	1 NEG			1 NEG	1 NEG	1			UNDETER	3 POS		2 POS		2	312.85	1.7948	1
4655 2388H1G	49	1 BIHAR	22-03-2016	1 4|8	1	1	0 44|F	WIFE	ADPKD	9 TG/ATN/P	3 SLOW GR/	3 NEG			1 NEG	1 NEG	1			POS	2 POS		2 POS		2	245.23	9.9772	1
4659 3498B3G	29	1 BANGLAD	31-03-2016	1 4|8	5	1	0 45|F	MOTHER	UNKNOWI	1 ACR	2			NEG	1 NEG	1 NEG	1			UNDETER	3 POS		2 POS		2	1202.07	2.1958	1
5165 7376J1G	25	2 ORISSA	16-02-2017	1 4|8		1	0 45|F	MOTHER	IgA;NEPHF	3 ACR	2			NEG	1 NEG	1 NEG	1	NEG	POS	POS	2 POS		2 POS		2	909.23	2.344	1
5355 0193B2G	33	1 TRIPURA	20-07-2017	1 6|8	7	1	0 54|M	FATHER	UNKNOWI	3 ACR+AMR 2+1				NEG	1 NEG	1 NEG	1 NON DSA	(Class I&II)	POS	2 POS		2 POS		2	120.05	3.406	1
5392 4942F8G	47	1 BIHAR	23-08-2017	1 3|8		1	0 47|F	UNRELATE	Cardiolus re	10 ACR	2			NEG	1 NEG	1 BP	3			POS	2 POS		2 POS		2	233.73	37.6582	1
5393 5325J1G	28	1 WEST BEN	23-08-2017	1 3|8	4	1	0 40|F	UNRELATE	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			UNDETER	3 POS		2 POS		2	312.17	48.7568	1
4891 5217A4G	24	1 TRIPURA	01-12-2016	1 4|8	2	1	0 47|F	MOTHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 BP (708)	3	NEG	POS		2 NEG		1 POS		2	40.36	1.5701	1
4725 2233B0G	41	1 BIHAR	10-05-2016	1 4|8		1	0 55|F	MOTHER	OBSTRUCT	10 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 UNDETER		3 POS		2	30.02	75.4504	1
4735 9845E2F	27	1 WEST BEN	18-05-2016	1 4|8	1	1	0 41|F	MOTHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			NEG	1 NEG		1 NEG		1	458.16	0.6904	1
4230 1260B9G	24	2 BANGLAD	23-06-2015	1 5|8		1	0 47|F	MOTHER	UNKNOWI	3 TG/ATN/P	3 A/C Graft	1 NEG			1 NEG	1 NEG	1			POS	2 POS		2 POS		2	476.01	1.5882	1
4935 3570B3G	22	1 WEST BEN	05-10-2016	1 6|8		1	0 50|M	FATHER	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			POS	2 NEG		1 POS		2	6.6	1.9777	1
5174 4835A4G	34	1 JHARKHAI	29-03-2017	6|8	2	1	0 30|F	WIFE	UNKNOWI	3 ACR	2			NEG	1 NEG	1 NEG	1			UNDETER	3 UNDETER		3 POS		2	2053	1.7118	1

DATA SPREAD SHEETS (CONTROLS)

SERUM I.N	Hosp No	AGE	GENDER_1M_2F	Trx date	Type_1L_	HLA MAT	TRANSFU	NO. OF TI	PREGNA	DONOR	RELATION	NKD	NKD_1	gABiopsy	Biopsy_r	Clinical D	cRIDiag_r	CDC	DSA	CLASSA	CLASSB	CLASSAB	FLOW	CLASS I	CLASS 1	CLASS II	CLASS II_	MICA	PC/NC	NBGRadi	group
4970	6067276	46	1.BIHAR	10-11-2016	1.B8			1	0.32	F	wife	DIABETIC	5			stable		NEG	1.NEG	1				NEG	1.NEG	1.UNDETERI		3	284.55	1.405	2
4980	6348316	43	2.CHATTISG	15-11-2016	1.7	8	3	1	3.41	M	Brother	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.UNDETERI	3.POS		2	410.16	2.792	2
4990	6112836	53	1.CHATTISG	29-11-2016	1.1	8		1	0.40	F	wife	UNKNOWI	3			stable		NEG	1.NEG	1				UNDETERM	3.NEG	1.POS		2	479.14	2.347	2
4408	1917946	33	1.JHARKHAI	17-12-2015	1.2	8		1	0.50	F	Mother	TMA SECC	11			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	196.13	2.065	2
4559	3201126	32	1.JHARKHAI	02-03-2016	1.2	8		1	0.24	F	WIFE	CTN	12			stable		NEG	1.NEG	1				NEG	1.NEG	1.NEG		1	238.84	0.631	2
4621	9123976	26	1.JHARKHAI	10-03-2016	1.4	8		1	0.49	F	MATERNA	IgA NEPH	1			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	268.24	1.765	2
4698	6141406	23	2.JHARKHAI	20-04-2016	1.4	8		1	0.53	F	MOTHER	IgA NEPH	1			stable		NEG	1.NEG	1				POS	2.UNDETERI	3.NEG		1	160.49	1.103	2
4743	2918506	37	1.BANGLAD	26-05-2016	1.2	8		1	0.23	F	wife	OBSTRUC	10			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	316.01	2.859	2
4776	3901096	60	1.WEST BEN	15-06-2016	1.6	8		1	0.26	M	NEPHEW	IgA NEPH	1			stable		NEG	1.NEG	1				POS	2.UNDETERI	3.POS		2	161.37	2.8082	2
4838	2471906	32	1.BANGLAD	10-08-2016	1.8	8	8	1	0.43	F	Sister	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.NEG		1	356.28	1.131	2
4862	2358976	21	2.JHARKHAI	18-08-2016	1.4	8	4	1	0.50	F	MOTHER	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	401.56	1.7702	2
3418	7008096	22	1.KERALA	10-04-2014	1.4	8	8	1	0.37	F	Mother	OBSTRUC	10			stable		NEG	1.NEG	1				POS	2.NEG	1.NEG		1	1672.16	1.0594	2
3757	6022335	22	2.TAMILNAI	14-10-2014	1.4	8		1	1.45	F	Mother	UNKNOWI	3			stable		NEG	1.NEG	1				UNDETERM	3.NEG	1.POS		2	2452.97	2.7322	2
3758	4505396	40	1.WEST BEN	16-10-2014	1.4	8	1	1	0.29	F	wife	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.UNDETERI	3.UNDETERI		3	768.32	1.469	2
3930	1110966	37	1.BHUTAN	28-01-2015	1.5	8		1	0.21	M	Brother	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	303.01	5.412	2
4168	4926810	44	2.BHUTAN	18-05-2015	1.4	8		1	3.33	M	Brother	MESANGU	13			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	317.66	1.703	2
4254	1587226	25	1.JHARKHAI	08-07-2015	1.5	8	1	1	0.46	F	Mother	IgA NEPH	1			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	624.26	4.1036	2
4278	2394946	50	1.MIZORAM	19-08-2015	1.4	8	1	1	0.42	F	wife	UNKNOWI	3			stable		NEG	1.NEG	1	B*(680)	3		UNDETERM	3.UNDETERI	3.NEG		1	1162.75	1.186	2
4411	0353026	35	2.CHATTISG	20-10-2015	1.1	8		1	0.34	M	Husband	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.NEG	1.POS		2	349.4	1.8724	2
4451	3122836	49	2.BHUTAN	17-11-2015	1.4	8		1	3.22	F	daughter	UNKNOWI	3			stable		NEG	1.NEG	1				UNDETERM	3.NEG	1.NEG		1	916.57	0.8652	2
4452	2723096	26	1.CHATTISG	25-11-2015	1.4	8	3	1	0.49	F	Mother	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	283.32	6.0688	2
4466	2899456	49	2.MADHYA	26-11-2015	1.4	8	2	1	2.52	F	Sister	LUPUS NE	14			stable		NEG	1.NEG	1				NEG	1.POS	2.POS		2	728.19	2.5972	2
4499	3617256	34	2.ASSAM	15-12-2015	1.4	8	2	1	0.47	M	COUSIN BI	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	533.05	1.746	2
4354	1774796	39	1.BANGLAD	08-09-2015	1.3	8	6	1	0.31	F	wife	UNKNOWI	3			stable		NEG	1.NEG	1	B*(878)	3		POS	2.POS	2.POS		2	324.18	3.378	2
4631	9778296	26	2.UTTARAKH	17-03-2016	1.4	8		1	0.47	F	Mother	UNKNOWI	3			stable		NEG	1.NEG	1				NEG	1.UNDETERI	3.NEG		1	650.93	1.0066	2
4979	6480666	24	1.WEST BEN	17-11-2016	1.4	8		1	0.43	F	Mother	IgA NEPH	1			stable		NEG	1.NEG	1	B*(547)	3		NEG	1.POS	2.NEG		1	50.29	0.4656	2
5119	7419946	46	2.CHATTISG	23-02-2017	1.4	8	2	1	2.51	M	Brother	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	657.56	2.0168	2
5132	7482966	21	1.WEST BEN	22-03-2017	1.7	8		1	0.50	F	Mother	IgA NEPH	1			stable		NEG	1.NEG	1				UNDETERM	3.NEG	1.NEG		1	302.33	1.0768	2
5253	7454196	21	2.KERALA	16-05-2017	1.5	8	1	1	0.45	F	Mother	ARPKD	15			stable		NEG	1.NEG	1			NEG	NEG	1.POS	2.NEG		1	493.99	0.919	2
3809	8027236	28	2.CHATTISG	12-11-2014	1.6	8	3	1	0.29	M	Husband	UNKNOWI	3			stable		NEG	1.NEG	1				NEG	1.NEG	1.NEG		1	129.04	0.1607	2
3897	0786506	44	2.BHUTAN	07-01-2015	1.4	8		1	2.20	M	SON	UNKNOWI	3			stable		NEG	1.NEG	1	B*(863)	3		NEG	1.NEG	1.POS		2	228.06	1.7511	2
3913	0800626	29	1.BHUTAN	20-01-2015	1.3	8		1	0.29	M	COUSIN BI	CTN	12			stable		NEG	1.NEG	1				NEG	1.POS	2.UNDETERI		3	320.68	1.282	2
4127	7331406	57	1.WEST BEN	30-04-2015	1.4	8		1	0.47	F	Sister	ARTERIOS	4			stable		NEG	1.NEG	1	B*(820)	3	NEG	NEG	1.NEG	1.NEG		1	240.31	0.1087	2
4552	7175146	29	1.WEST BEN	03-02-2016	1.4	8		1	0.55	F	Mother	IgA NEPH	1			stable		NEG	1.NEG	1				NEG	1.NEG	1.NEG		1	287.31	0.3264	2
4674	9956766	40	1.NEPAL	14-04-2016	1.1	8	3	1	0.38	F	wife	UNKNOWI	3			stable		NEG	1.NEG	1				NEG	1.POS	2.NEG		1	201.51	1.148	2
4709	3685346	59	2.BHUTAN	26-04-2016	1.5	8	3	1	1.29	M	NEPHEW	UNKNOWI	3			stable		NEG	1.NEG	1				NEG	1.NEG	1.UNDETERI		3	213.72	1.2107	2
4810	1034676	31	2.MIZORAM	13-07-2016	1.6	8	2	1	0.30	M	COUSIN BI	LUPUS NE	14			stable		NEG	1.NEG	1				NEG	1.UNDETERI	3.UNDETERI		3	300.75	1.2066	2
4875	6490656	62	2.CHATTISG	20-08-2016	2.0	8		1	2.49	M	unrelated	UNKNOWI	3			stable		NEG	1.NEG	1				NEG	1.NEG	1.POS		2	199.18	4.9231	2
4871	4691576	32	2.NAGALAN	09-01-2016	1.7	8		1	0.55	F	Mother	UNKNOWI	3			stable		NEG	1.NEG	1	B*(852)	3		NEG	1.NEG	1.NEG		1	112.89	0.4236	2
5133	2356626	32	1.WEST BEN	28-02-2017	1.4	8		1	0.62	F	Mother	IgA NEPH	1			stable		NEG	1.NEG	1			NEG	NEG	1.NEG	1.POS		2	285.26	3.4372	2
4215	7792626	25	2.JHARKHAI	06-11-2015	1.3	8	1	1	0.51	M	Brother	IgA NEPH	1			stable		NEG	1.NEG	1				NEG	1.NEG	1.POS		2	85.31	8.6908	2
4335	8624456	42	1.JHARKHAI	26-08-2015	1.3	8	1	1	0.37	F	wife	DIABETIC	5			stable		NEG	1.NEG	1				NEG	1.NEG	1.UNDETERI		3	341.53	1.7613	2
4487	3308666	54	2.BHUTAN	09-12-2015	1.4	8		1	6.27	M	SON	UNKNOWI	3			stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	130.14	3.2445	2
4777	8514446	27	2.TAMILNAI	14-06-2016	1.5	8		1	0.51	F	Mother	CIN/OBST	12	10		stable		NEG	1.NEG	1				POS	2.POS	2.POS		2	67.36	3.728	2

Pre transplant MICA antibodies and impact on renal allograft outcome

Study ID number: Date of entry:

Date of renal transplantation: Unit:

Native Kidney Disease:

	RECIPIENT	DONOR
NAME		
HOSP NO		
AGE & SEX		
Address		
BLOOD GROUP		
HLA A	A* A*	A* A*
B	B* B*	B* B*
DR	DRB1* DRB1*	DRB1* DRB1*
DQ	DQB1* DQB1*	DQB1* DQB1*
Blood transfusion	Internal : External:	Relationship:
Height (cm)/ Weight (kg)	cm / Kg	

HIV: NEG HBV: NEG HCV: NEG

History of Preeclampsia: NA

HTN: DM: OTHER COMORBIDITY:

HLA Crossmatch (CDC)

Date		
Regular /DTT	Neg	
Auto	Neg	
Routine	Neg	
Extended	Neg	

Pre transplant MICA antibodies and impact on renal allograft outcome

DSA Class I: Neg (MFI:) Class II: Neg (MFI:) Date:

:

Induction regimen: IL2RB ATG

Immunosuppressive regimen used:

(Prednisolone + Tacrolimus + Mycophenolate)

Any event leading to reduction in immunosuppression:

(If yes specify the reason)

Drug adverse effect: ☐ Infection: ☐ Others: ☐

Presence of perioperative Events:

Post-operative events:

Post transplantation period:

	Wt (kg)	BP (mm Hg)	Temp	Urea	Creatinine	TC/mm3
			N			
			N			
			N			
			N			
			N			
			N			

For patients went into graft rejection

Acute Cell Mediated Rejection ☐ Antibody Mediated Rejection ☐

Chronic rejection ☐

Status at the time of rejection:

How many days/weeks/months post transplantation?

Creatinine :

Pre transplant MICA antibodies and impact on renal allograft outcome

Immunosuppression status:

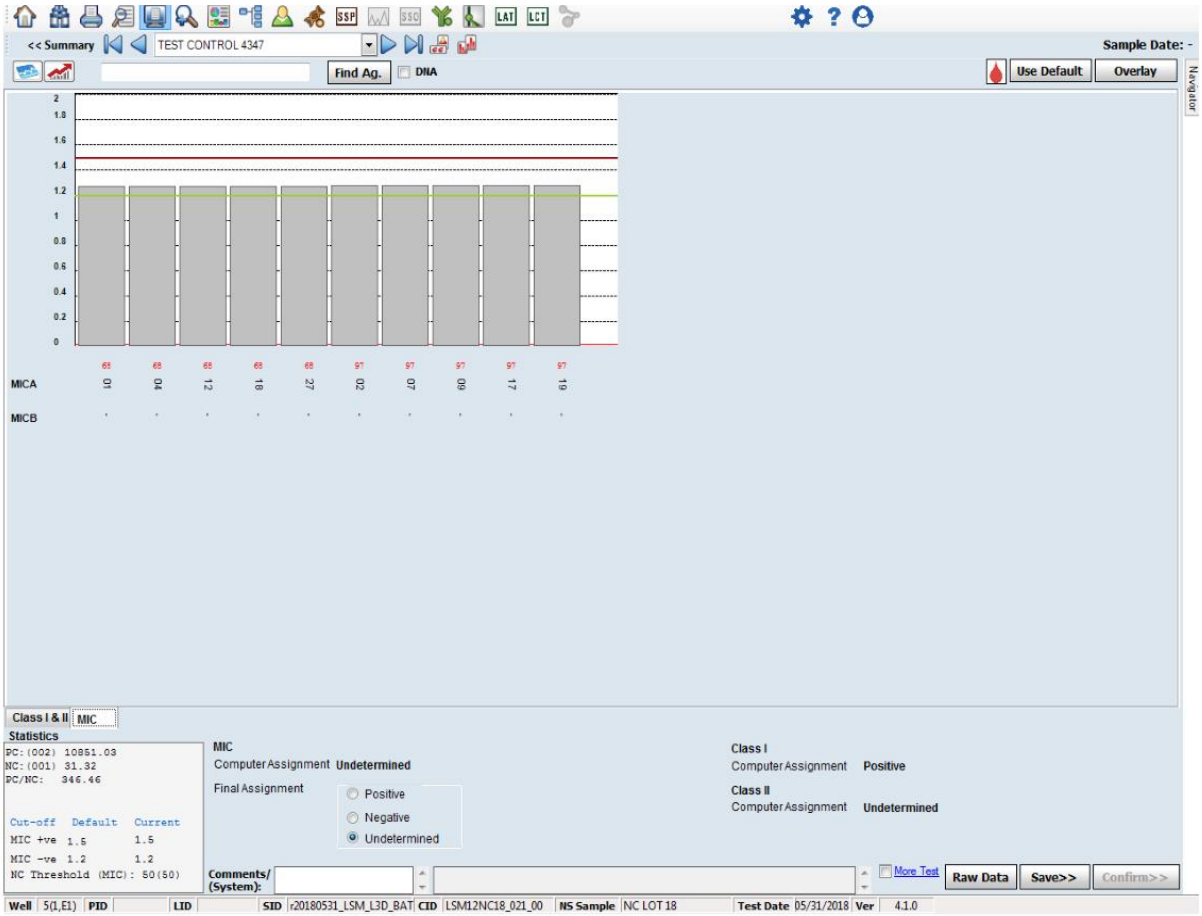
Is rejection Biopsy Proven?

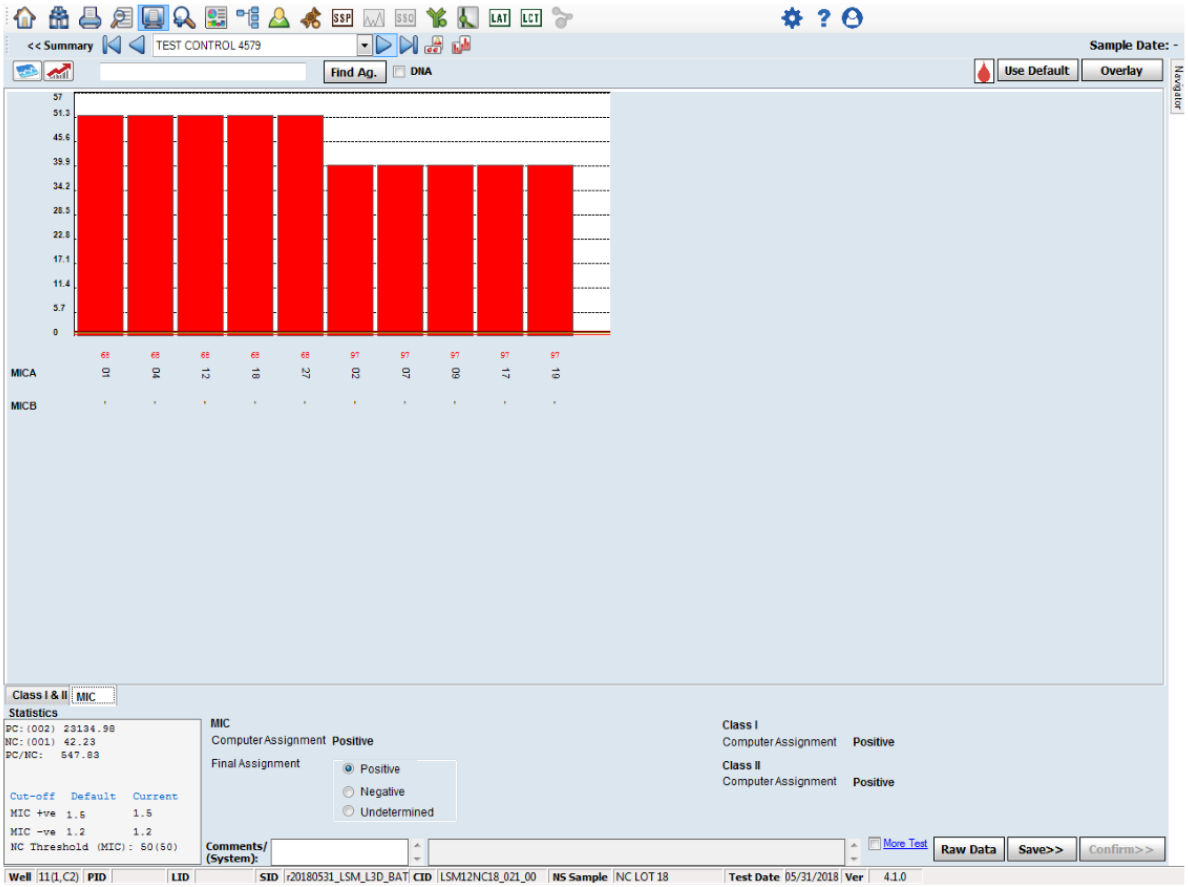
DSA Positive Negative

Treatment given:

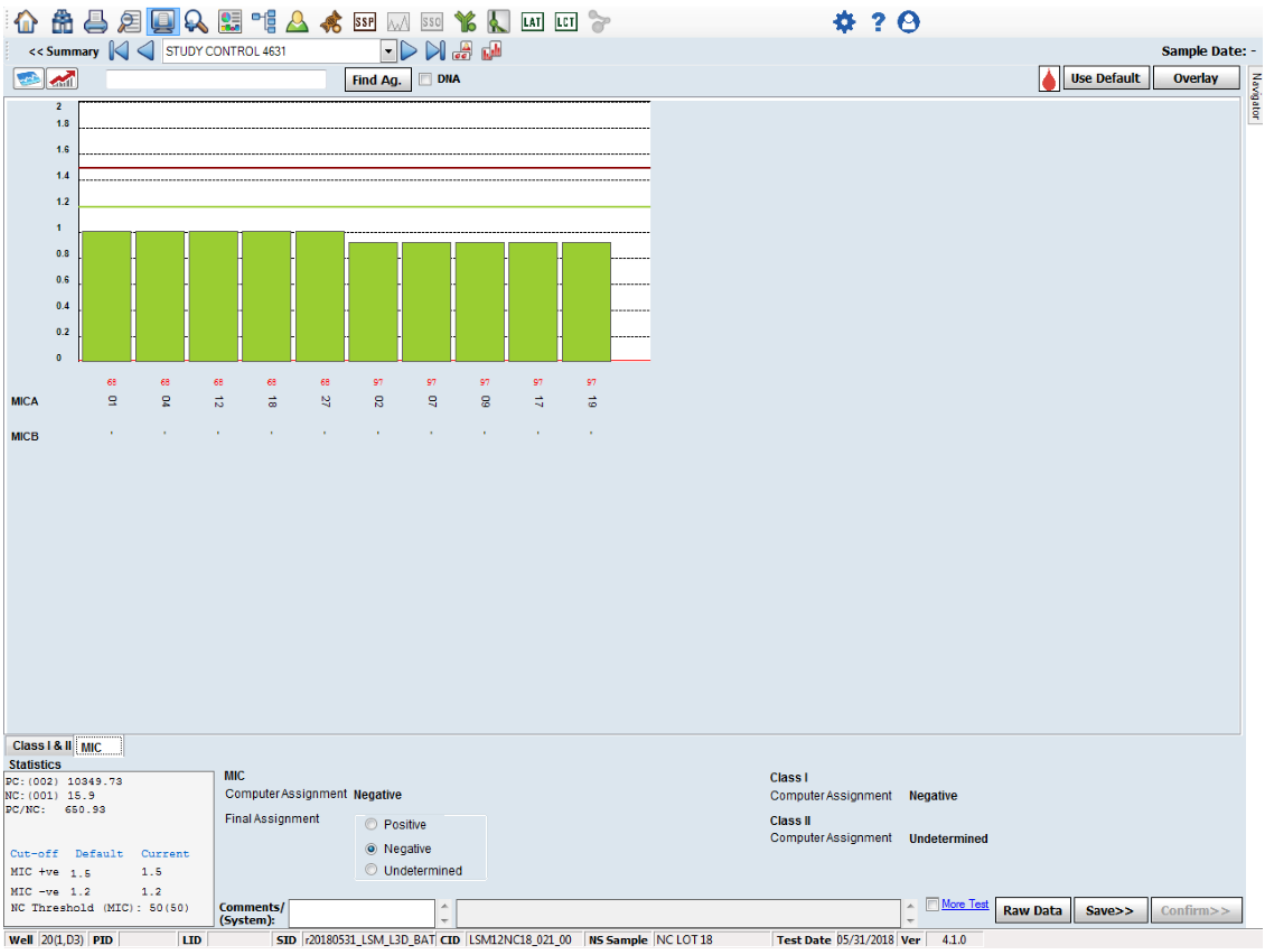
Pre transplantation Anti MICA antibody

MIC ANALYSIS HISTOGRAM: UNDETERMINED





MIC ANALYSIS HISTOGRAM: POSITIVE



MIC ANALYSIS HISTOGRAM: NEGATIVE